



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US98/10842 <b>(22) International Filing Date:</b> 28 May 1998 (28.05.98) <b>(30) Priority Data:</b> 60/048,290 2 June 1997 (02.06.97) US <b>(71) Applicant:</b> ZYMOGENETICS, INC. [US/US]; 1201 Eastlake Avenue East, Seattle, WA 98102 (US). <b>(72) Inventor:</b> SHEPPARD, Paul, O.; 20717 N.E. 2nd Street, Redmond, WA 98053 (US). <b>(74) Agent:</b> LINGENFELTER, Susan, E.; ZymoGenetics, Inc., 1201 Eastlake Avenue East, Seattle, WA 98102 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>																																				
<b>(54) Title:</b> NEUROKININ B PRECURSORS  <table><tr><td>TKNK_MOUSE</td><td>MRSAMLFAAV LALSLAWTFG AVCEEPQEQ. ...GGRLSKD SDLYQLPPSL</td><td>: 46</td></tr><tr><td>TKNK_RAT</td><td>MRSAMLFAAV LALSLAWTFG AACEEPQEQ. ....GGRLSKD SDLSLLPPPL</td><td>: 46</td></tr><tr><td>TKNK_BOVIN</td><td>MRSTLLFAVI LALSSARSLG AVCEESQEQV VPGGGHKKD SNLYQLPPSL</td><td>: 50</td></tr><tr><td>zneurok1</td><td>MRIMLLFTAI LAFSLAQSG AVCKEPQEEV VPGGGRSKRD PDLYQ....L</td><td>: 46</td></tr><tr><td>TKNK_MOUSE</td><td>LRRLYDSRPV SLEGLLKVLS KASVGPKETS LPQKRDMHDF FVGLMGKRNS</td><td>: 96</td></tr><tr><td>TKNK_RAT</td><td>LRRLYDSRSI SLEGLLKVLS KASVGPKETS LPQKRDMHDF FVGLMGKRNS</td><td>: 96</td></tr><tr><td>TKNK_BOVIN</td><td>LRRLYDSRVV SLDGLLKMLS KASVGPKESP LPQKRDMHDF FVGLMGKRNL</td><td>: 100</td></tr><tr><td>zneurok1</td><td>LQRLFKSHS. SLEGLLKALS QASTDPKEST SPEKRDMHDF FVGLMGKRSV</td><td>: 95</td></tr><tr><td>TKNK_MOUSE</td><td>QPDTPTDVVE ENTPSFGILK ~~~~~</td><td>: 116</td></tr><tr><td>TKNK_RAT</td><td>QPDTPADVVE ENTPSFGVLK ~~~~~</td><td>: 116</td></tr><tr><td>TKNK_BOVIN</td><td>QPDTPVDINQ ENIPSFGTFK YPPSVE</td><td>: 126</td></tr><tr><td>zneurok1</td><td>QPDSPTDVNQ ENVPSFGILK YPPRAE</td><td>: 121</td></tr></table> <b>(57) Abstract</b> <p>The present invention relates to zneurok1 polypeptides and polynucleotides encoding the same. These polypeptides are novel members of a family of proteins that are precursors of neurokinin B, a ten amino acid moiety of biological significance. The polypeptides, and polynucleotides encoding them, are useful in the study of prohormone convertase function and neurokinin receptors. The present invention also includes antibodies to the zneurok1 polypeptides.</p>			TKNK_MOUSE	MRSAMLFAAV LALSLAWTFG AVCEEPQEQ. ...GGRLSKD SDLYQLPPSL	: 46	TKNK_RAT	MRSAMLFAAV LALSLAWTFG AACEEPQEQ. ....GGRLSKD SDLSLLPPPL	: 46	TKNK_BOVIN	MRSTLLFAVI LALSSARSLG AVCEESQEQV VPGGGHKKD SNLYQLPPSL	: 50	zneurok1	MRIMLLFTAI LAFSLAQSG AVCKEPQEEV VPGGGRSKRD PDLYQ....L	: 46	TKNK_MOUSE	LRRLYDSRPV SLEGLLKVLS KASVGPKETS LPQKRDMHDF FVGLMGKRNS	: 96	TKNK_RAT	LRRLYDSRSI SLEGLLKVLS KASVGPKETS LPQKRDMHDF FVGLMGKRNS	: 96	TKNK_BOVIN	LRRLYDSRVV SLDGLLKMLS KASVGPKESP LPQKRDMHDF FVGLMGKRNL	: 100	zneurok1	LQRLFKSHS. SLEGLLKALS QASTDPKEST SPEKRDMHDF FVGLMGKRSV	: 95	TKNK_MOUSE	QPDTPTDVVE ENTPSFGILK ~~~~~	: 116	TKNK_RAT	QPDTPADVVE ENTPSFGVLK ~~~~~	: 116	TKNK_BOVIN	QPDTPVDINQ ENIPSFGTFK YPPSVE	: 126	zneurok1	QPDSPTDVNQ ENVPSFGILK YPPRAE	: 121
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Description

## NEUROKININ B PRECURSORS

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## BACKGROUND OF THE INVENTION

Mammalian neurokinins (also referred to as tachykinins) are small peptides that appear to be involved in numerous physiological functions. Such neurokinins include substance P (SP), an 11 amino acid polypeptide; neurokinin A (NKA, also referred to as neuromedin L and substance K), a 10 amino acid polypeptide; and neurokinin B (NKB, also known as neuromedin K, neuromedin B and neurokinin K), a ten amino acid polypeptide. Three mammalian neurokinin receptors have been identified, each with a characteristic neurokinin binding preference pattern. Receptor NK1 exhibits a preference for SP, but also interacts with NKB and NKA. See, for example, Maggi, General Pharmacology (United Kingdom) 26(5): 911-44, 1995. Receptor NK2 exhibits a preference for NKA, but also interacts with NKB and SP. See, for example, Huber et al., Eur. J. Pharmacol. (Netherlands) 239(1-3): 103-9, 1993. Receptor NK3 exhibits a preference for NKB, but also interacts with NKA and SP. See, for example, Maggi et al., Regulatory Peptides 53: 259-74, 1994.

Mammalian neurokinins are generally expressed in the form of precursor proteins. Cleavage of precursor proteins releases active neurokinins. A bovine NKB precursor protein is described in Kotani et al., Proc. Natl. Acad. Sci. (USA) 83: 7074-8, 1986. The deduced amino acid sequence of the disclosed bovine NKB precursor is 126 amino acid residues long with a putative signal sequence at the 5' end thereof. Two NKB precursor-encoding mRNAs were discovered, differing only in the 5' untranslated region. The more abundant mRNA is encoded by seven exons. In contrast, the less abundant mRNA includes two additional 5' exons and does not include the 5'

terminus of the first exon of the more abundant mRNA. This gene organization resembles that of a precursor protein encoding SP and NKA; however, the major expression sites differ substantially. The two genes are believed to originate from a common ancestor gene, with diversity acquired through cellular mechanisms, such as gene duplication and differential expression of duplicated genes.

Neurokinins have been implicated in a number of physiological processes. Such processes include neurotransmission/neuromodulation in the nervous system and peripheral tissues, smooth muscle contraction (e.g., in respiratory, gastrointestinal and urinary tissue), growth/proliferation (e.g., small cell carcinoma), hormone secretion (e.g., pancreas, pituitary gland and gastrin-secreting cells), inhibition of gastric emptying, modulation of neutrophil function, blood pressure regulation and the like. See, for example, Kotani et al. (referenced above); Belloli et al., J. Vet. Pharmacol. Therap. 17: 379-83, 1994; Battey et al., Journal of the National Cancer Institute Monographs 13: 141-4, 1992; Henriksen et al., J. of Receptor & Signal Transduction Research 15(1-4): 529-41, 1995; Dobrzanski et al., Regulatory Peptides 45: 341-52, 1993; Varga et al., Eur. J. Pharmacology 286: 109-112, 1995; Wozniak et al., Immunology 78: 629-34, 1993; and Muneakata, Comp. Biochem. Physiol. 98C(1): 171-9, 1991. Antibodies directed to NKB precursor protein and to the NKB-preferring NK3 receptor, for example, have been prepared. See, for example, Muneakata (referenced above, antibody to a proneurokinin B protein) and Ding et al., J. Comparative Neurology 364: 290-310, 1996 (antibody to the NK3 receptor).

Neurokinins are generally expressed as precursor molecules encompassing the active proteins. Evidence exists that precursor proteins can be more effective upon administration than active protein alone. Polypeptide

precursors of neurokinins are therefore sought for the study of neurokinin-related physiological processes. The present invention provides such polypeptides for these and other uses that should be apparent to those skilled in the art from the teachings herein.

#### SUMMARY OF THE INVENTION

Within one aspect the invention provides an isolated polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of residue 17, 19 or 21 to residue 121 of SEQ ID NO. 2, and wherein said polypeptide releases a neurokinin B polypeptide in the presence of a prohormone convertase capable of cleaving dibasic amino acids. Within a related embodiment is provided a polypeptide having the amino acid sequence of amino acid residue 17, 19 or 21 to amino acid residue 121 of SEQ ID NO. 2. Within another embodiment is provided a polypeptide having the amino acid sequence of amino acid residue 1 or 4 to amino acid residue 121 of SEQ ID NO:2.

Within another aspect is provided a pharmaceutical composition comprising a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of residue 17, 19 or 21 to residue 121 of SEQ ID NO. 2, and wherein said polypeptide releases a neurokinin B polypeptide in the presence of a prohormone convertase capable of cleaving dibasic amino acids; in combination with a pharmaceutically acceptable vehicle.

Within another aspect is provided an antibody that specifically binds to an epitope of a polypeptide of SEQ ID NO:2.

Within another aspect is provided an anti-idiotypic antibody of an antibody which specifically binds to an epitope of a polypeptide of SEQ ID NO:2.

Within another aspect is provided a binding protein which specifically binds to an epitope of a polypeptide of SEQ ID NO:2.

Within yet another aspect is provided a fusion protein consisting essentially of a first portion and a second portion joined by a peptide bond, said first portion comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of residue 17, 19 or 21 to residue 121 of SEQ ID NO. 2, and wherein said polypeptide releases a neurokinin B polypeptide in the presence of a prohormone convertase capable of cleaving dibasic amino acids; and said second portion consisting essentially of a second polypeptide.

Within another aspect is provided a fusion protein comprising a secretory signal sequence selected from the group consisting of: (a) amino acid residues 1-16 of SEQ ID NO:2; (b) amino acid residues 1-18 of SEQ ID NO:2; (c) amino acid residues 1-20 of SEQ ID NO:2; (d) amino acid residues 4-16 of SEQ ID NO:2; (e) amino acid residues 4-18 of SEQ ID NO:2; and (f) amino acid residues 4-10 of SEQ ID NO:2; wherein said secretory signal sequence is operably linked to an additional polypeptide.

Within another aspect is provided an isolated polynucleotide molecule encoding a polypeptide as described above. Within one embodiment is provided a polynucleotide molecule encoding a polypeptide having the amino acid sequence of amino acid residue 17, 19 or 21 to amino acid residue 121 of SEQ ID NO. 2. Within another embodiment is provided a polynucleotide encoding a polypeptide having the amino acid sequence of amino acid residue 1 or 4 to amino acid residue 121 of SEQ ID NO:2.

Within another aspect is provided an isolated polynucleotide molecule selected from the group consisting of: (a) DNA molecules encoding a neurokinin B precursor polypeptide comprising a nucleotide sequence as shown in SEQ ID NO:1 from nucleotide 137, 146, 185, 191 or 197 to

nucleotide 812; (b) DNA molecules encoding a neurokinin B precursor polypeptide which are at least 85% identical in nucleotide sequence to (a); (c) degenerate nucleotide sequences of (a) or (b); and molecules complementary to  
5 (a), (b) or (c); wherein the polypeptides release a neurokinin B polypeptide in the presence of a prohormone convertase capable of cleaving dibasic amino acids.

Within another aspect is provided a polynucleotide encoding a human zneurok1 polypeptide  
10 fragment, said polypeptide fragment further comprising an N-terminal and a C-terminal proteolytic cleavage site cleavable by a prohormone convertase. Within one embodiment the polynucleotide is selected from the group consisting of: (a) nucleotide 377 to 406 of SEQ ID NO:1;  
15 (b) nucleotide 392 to 406 of SEQ ID NO:1; (c) nucleotide 368 to 418 of SEQ ID NO:1; (d) nucleotide 392 to 418 of SEQ ID NO:1; (e) nucleotide 254 to 289 of SEQ ID NO:1; and (e) nucleotide 245 to 298 of SEQ ID NO:1. Within another embodiment the N-terminal and C-terminal cleavage sites  
20 are independently dibasic cleavage sites selected from the group consisting of: (a) lys-lys; (b) arg-arg; (c) lys-arg; and (d) arg-lys.

Within another aspect is provided an isolated polynucleotide molecule encoding a fusion protein  
25 consisting essentially of a first portion and a second portion joined by a peptide bond, said first portion comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of residue 17, 19 or 21 to residue 121 of SEQ ID NO. 2, and wherein said  
30 polypeptide releases a neurokinin B polypeptide in the presence of a prohormone convertase capable of cleaving dibasic amino acids; and said second portion consisting essentially of a second polypeptide.

Within another aspect is provided an isolated  
35 polynucleotide molecule encoding a fusion protein comprising a secretory signal sequence selected from the



group consisting of: (a) amino acid residues 1-16 of SEQ ID NO:2; (b) amino acid residues 1-18 of SEQ ID NO:2; (c) amino acid residues 1-20 of SEQ ID NO:2; (d) amino acid residues 4-16 of SEQ ID NO:2; (e) amino acid residues 4-18 of SEQ ID NO:2; and (f) amino acid residues 4-10 of SEQ ID NO:2; wherein said secretory signal sequence is operably linked to an additional polypeptide.

Within yet another aspect is provided an expression vector comprising the following operably linked elements: a transcription promoter; a DNA segment encoding a polypeptide as described above; and a transcription terminator. Within one embodiment the DNA segment further encodes a secretory signal sequence operably linked to said polypeptide. Within another embodiment the secretory signal sequence is selected from the group consisting of: (a) amino acid residues 1-16 of SEQ ID NO:2; (b) amino acid residues 1-18 of SEQ ID NO:2; (c) amino acid residues 1-20 of SEQ ID NO:2; (d) amino acid residues 4-16 of SEQ ID NO:2; (e) amino acid residues 4-18 of SEQ ID NO:2; and (f) amino acid residues 4-10 of SEQ ID NO:2.

Within another aspect is provided a cultured cell into which has been introduced an expression vector comprising the following operably linked elements: a transcription promoter; a DNA segment encoding a polypeptide as described above; and a transcription terminator; wherein the cell expresses the polypeptide encoded by the DNA segment.

Within another aspect is provided a method of producing a polypeptide comprising: culturing a cell into which has been introduced an expression vector comprising the following operably linked elements: a transcription promoter; a DNA segment encoding a polypeptide as described above; and a transcription terminator; whereby said cell expresses said polypeptide encoded by said DNA segment; and recovering said expressed polypeptide.



Within another aspect is provided a method for detecting a genetic abnormality in a patient, comprising: obtaining a genetic sample from a patient; incubating the genetic sample with a polynucleotide comprising at least 5 14 contiguous nucleotides of SEQ ID NO:1 or the complement of SEQ ID NO:1, under conditions wherein said polynucleotide will hybridize to complementary polynucleotide sequence, to produce a first reaction product; comparing said first reaction product to a 10 control reaction product, wherein a difference between said first reaction product and said control reaction product is indicative of a genetic abnormality in the patient.

#### 15 BRIEF DESCRIPTION OF THE DRAWINGS

The Figure illustrates a multiple alignment of three members of the neurokinin B precursor family of polypeptides and a zneurok1 polypeptide of the present invention. The three family members are as follows: 20 TKNK\_Mouse (Kako et al., Biomed. Res. 14: 253-9, 1993); TKNK\_Rat (Kako et al. ibid.); and TKNK\_Bovin (Kotani et al. Proc. Natl. Acad. Sci. (USA) 83: 7074-78, 1986 and Kako et al. ibid.).

#### 25 DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention in detail, it may be helpful to the understanding thereof to define the following terms:

30 The term "affinity tag" is used herein to denote a peptide segment that can be attached to a polypeptide to provide for purification of the polypeptide or provide sites for attachment of the polypeptide to a substrate. In principal, any peptide or protein for which an antibody 35 or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-

histidine tract, Glu-Glu (Grussenmeyer et al. Proc. Natl. Acad. Sci. USA 82:7952-4, 1995), protein A (Nilsson et al., *EMBO J.* 4:1075, 1985; Nilsson et al., *Methods Enzymol.* 198:3, 1991), glutathione S transferase (Smith and Johnson, *Gene* 67:31, 1988), substance P, Flag<sup>TM</sup> peptide (Hopp et al., *Biotechnology* 6:1204-1210, 1988; available from Eastman Kodak Co., New Haven, CT), streptavidin binding peptide, or other antigenic epitope or binding domain. See, in general Ford et al., *Protein Expression and Purification* 2: 95-107, 1991. DNAs encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ).

The term "allelic variant" denotes any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequence. The term allelic variant is also used herein to denote a protein encoded by an allelic variant of a gene.

The terms "amino-terminal" and "carboxyl-terminal" are used herein to denote positions within polypeptides and proteins. Where the context allows, these terms are used with reference to a particular sequence or portion of a polypeptide or protein to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a protein is located proximal to the carboxyl terminus of the reference sequence, but is not necessarily at the carboxyl terminus of the complete protein.

The term "complement/anti-complement pair" denotes non-identical moieties that form a non-covalently associated, stable pair under appropriate conditions. For instance, biotin and avidin (or streptavidin) are

prototypical members of a complement/anti-complement pair. Other exemplary complement/anti-complement pairs include receptor/ligand pairs, antibody/antigen (or hapten or epitope) pairs, sense/antisense polynucleotide pairs, and  
5 the like. Where subsequent dissociation of the complement/anti-complement pair is desirable, the complement/anti-complement pair preferably has a binding affinity of  $<10^9 \text{ M}^{-1}$ .

The term "degenerate nucleotide sequence"  
10 denotes a sequence of nucleotides that includes one or more degenerate codons (as compared to a reference polynucleotide molecule that encodes a polypeptide). Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (i.e.,  
15 GAU and GAC triplets each encode Asp).

The term "expression vector" denotes a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription.  
20 Such additional segments may include promoter and terminator sequences, and may optionally include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from  
25 plasmid or viral DNA, or may contain elements of both.

The term "isolated", when applied to a polynucleotide, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is  
30 in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes  
35 with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as

promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, Nature 316:774-78, 1985).

5 An "isolated" polypeptide or protein is a polypeptide or protein that is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated polypeptide is substantially free of other polypeptides,  
10 particularly other polypeptides of animal origin. It is preferred to provide the polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. When used in this context, the term "isolated" does not exclude the presence of the same  
15 polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

The term "operably linked", when referring to DNA segments, denotes that the segments are arranged so that they function in concert for their intended purposes,  
20 e.g. transcription initiates in the promoter and proceeds through the coding segment to the terminator.

The term "ortholog" denotes a polypeptide or protein obtained from one species that is the functional counterpart of a polypeptide or protein from a different  
25 species. Sequence differences among orthologs are the result of speciation.

The term "polynucleotide" denotes a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end.  
30 Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are expressed as base pairs (abbreviated "bp"), nucleotides ("nt"), or kilobases  
35 ("kb"). Where the context allows, the latter two terms may describe polynucleotides that are single-stranded or

double-stranded. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term "base pairs". It will be recognized by those skilled in the art  
5 that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired. Such unpaired ends will in  
10 general not exceed 20 nt in length.

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as  
15 "peptides".

"Probes and/or primers" as used herein can be RNA or DNA. DNA can be either cDNA or genomic DNA. Polynucleotide probes and primers are single or double-stranded DNA or RNA, generally synthetic oligonucleotides,  
20 but may be generated from cloned cDNA or genomic sequences or its complements. Analytical probes will generally be at least 20 nucleotides in length, although somewhat shorter probes (14-17 nucleotides) can be used. PCR primers are at least 5 nucleotides in length, preferably  
25 15 or more nt, more preferably 20-30 nt. Short polynucleotides can be used when a small region of the gene is targeted for analysis. For gross analysis of genes, a polynucleotide probe may comprise an entire exon or more. Probes can be labeled to provide a detectable  
30 signal, such as with an enzyme, biotin, a radionuclide, fluorophore, chemiluminescer, paramagnetic particle and the like, which are commercially available from many sources, such as Molecular Probes, Inc., Eugene, OR, and Amersham Corp., Arlington Heights, IL, using techniques  
35 that are well known in the art.

The term "promoter" denotes a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

A "protein" is a macromolecule comprising one or more polypeptide chains. A protein may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and will vary with the type of cell. Proteins are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

The term "receptor" denotes a cell-associated protein that binds to a bioactive molecule (i.e., a ligand) and mediates the effect of the ligand on the cell. Membrane-bound receptors are characterized by a multi-domain structure comprising an extracellular ligand-binding domain and an intracellular effector domain that is typically involved in signal transduction. Binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the effector domain and other molecule(s) in the cell. This interaction in turn leads to an alteration in the metabolism of the cell. Metabolic events that are linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids. Most nuclear receptors also exhibit a multi-domain structure, including an amino-terminal, transactivating domain, a DNA binding domain and a ligand binding domain. In general, receptors can be membrane bound, cytosolic or nuclear; monomeric (e.g., thyroid



stimulating hormone receptor, beta-adrenergic receptor) or multimeric (e.g., PDGF receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor).

5           The term "secretory signal sequence" denotes a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger peptide  
10 is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

          Molecular weights and lengths of polymers determined by imprecise analytical methods (e.g., gel electrophoresis) will be understood to be approximate  
15 values. When such a value is expressed as "about" X or "approximately" X, the stated value of X will be understood to be accurate to  $\pm 10\%$ .

          The present invention is based in part upon the discovery of a novel DNA sequence that encodes a  
20 polypeptide having homology to neurokinin B (NKB) precursor proteins previously identified in rats, mice and cows, including the conserved active NKB polypeptide at amino acids 81-90 of SEQ ID NO. 2.

          Analysis of the tissue distribution of the mRNA  
25 corresponding to this novel DNA showed that expression was highest in placenta, followed by apparent but much decreased expression levels in brain, small intestine, stomach and spinal cord. A single 1.2 kb transcript was observed. The polypeptide has been designated zneurok1.

30           The novel zneurok1 polypeptides of the present invention were initially identified by querying an EST database for secretory signal sequences, characterized by an upstream methionine start site, a hydrophobic region of approximately 13 amino acids and a cleavage site, in an  
35 effort to select for secreted proteins. Polypeptides corresponding to ESTs meeting those search criteria were



compared to known sequences to identify secreted proteins having homology to known ligands. A single EST sequence was discovered and predicted to be a secreted protein. In this case, three putative cleavage sites were identified, 5 between amino acid residues 16 (Ala) and 17 (Gln), between amino acid residues 18 (Ser) and 19 (Phe), and between amino acid residues 20 (Gly) and 21 (Ala) of SEQ ID NO. 2. Full length sequencing thereof allowed discovery of a homolog relationship to secreted NKB 10 precursor proteins found in rat, mouse and cow as shown in the Figure.

The full sequence of the zneurok1 polypeptide was obtained from a single clone believed to contain it, wherein the clone was obtained from a brain tumor tissue 15 library. Other libraries that might also be searched for such clones include brain, small intestine, placenta, stomach, spinal cord and the like.

The nucleotide sequence of the N-terminal EST is described in SEQ ID NO. 1, and its deduced amino acid 20 sequence is described in SEQ ID NO. 2. Alternative methionine start sites were identified at positions 1 and 4 of SEQ ID NO. 2. When the alternative start sites are considered in combination with the alternative cleavage sites, six possible signal sequences are identified as 25 follows:

- 1) Amino acid residues 1 (Met) through 16 (Ala) of SEQ ID NO:2, nucleotides 137-184 of SEQ ID NO:1;
- 2) Amino acid residues 1 (Met) through 18 (Ser) 30 of SEQ ID NO:2, nucleotides 137-190 of SEQ ID NO:1;
- 3) Amino acid residues 1 (Met) through 20 (Gly) of SEQ ID NO:2, nucleotides 137-196 of SEQ ID NO:1;

- 4) Amino acid residues 4 (Met) through 16 (Ala) of SEQ ID NO:2, nucleotides 146-184 of SEQ ID NO:1;
- 5) Amino acid residues 4 (Met) through 18 (Ser) of SEQ ID NO:2, nucleotides 146-190 of SEQ ID NO:1; and
- 6) Amino acid residue 4 (Met) through 20 (Gly) of SEQ ID NO:2, nucleotides 146-196 of SEQ ID NO:1.

Those skilled in the art will recognize that predicted secretory signal sequence domain boundaries are approximations based on primary sequence content, and may vary slightly; however, such estimates are generally accurate to within  $\pm 4$  amino acid residues. Therefore the present invention also includes the polypeptides having amino acid sequences comprising amino acid residues 13-121 of SEQ ID NO:2, residues 14-121 of SEQ ID NO:2, residues 15-121 of SEQ ID NO:2, residues 21-121 of SEQ ID NO:2, residues 22-121 of SEQ ID NO:2, residues 23-121 and residues 24-121 of SEQ ID NO:2 as well as the polynucleotides encoding them.

Zneurok1 polypeptide encodes active NKB at residues 81-90, nucleotides 377-406 of SEQ ID NO:1. The neurokinin protein family members share the sequence of SEQ ID NO. 3 at the carboxy terminal end thereof. More specifically, the neurokinins share a common carboxy terminus as follows:

Phe-Xaa-Gly-Leu-Met-NH<sub>2</sub>.

Also provided by the present invention are active fragments of the zneurok1 polypeptide which contain the neurokinin B polypeptide (NKB), as shown in SEQ ID NO. 2 from amino acid 81 (Asp) to 90 (Met) or the carboxy terminal portion thereof from amino acid 86 (Phe) to 90 (Met); and cleavage sites on the 5' and 3' ends of the NKB polypeptide to permit release of the active form thereof. Preferred active fragments of the present invention,

particularly useful with engineered cleavage sites of choice, ranges from amino acid 81 (Asp) to amino acid 90 (Met) or from amino acid 86 (Phe) to 90 (Met) of SEQ ID NO. 2. Such active fragments may be encoded, for example, by a polyoligonucleotide including nucleotides 377-406 or nucleotides 392-406 as shown in SEQ ID NO. 1. Another preferred fragment of the present invention, including native zneurok1 polypeptide cleavage sites ranges from amino acid 78 (Glu) to amino acid 94 (Ser) of SEQ ID NO. 2. Such active fragments may be encoded, for example, by a polynucleotide including nucleotides 368 to 418. Still another preferred active fragment of the present invention ranges from amino acid 86 (Phe) to amino acid 94 (Ser), nucleotides 392-418 of SEQ ID NO:1, and an engineered cleavage site at the 5' end. One of ordinary skill in the art would recognize that complementary or degenerate polynucleotide sequences could also be used in this embodiment of the present invention.

Also provided by the present invention are active fragments of the zneurok1 polypeptide which contain a polypeptide, as shown in SEQ ID NO. 2 from amino acid 40 (Asp) to 51 (Phe); and cleavage sites on the 5' and 3' ends thereof to permit release of the active form thereof. Preferred active fragments of the present invention, particularly useful with engineered cleavage sites of choice, ranges from amino acid 40 (Asp) to amino acid 51 (Phe) of SEQ ID NO. 2. Such active fragments may be encoded, for example, by a polyoligonucleotide including nucleotides 254-289 as shown in SEQ ID NO. 1. Another preferred fragment of the present invention, including native zneurok1 polypeptide cleavage sites ranges from amino acid 37 (Ser) to amino acid 54 (His) of SEQ ID NO. 2. Such active fragments may be encoded, for example, by a polynucleotide including nucleotides 245 to 298. One of ordinary skill in the art would recognize that

complementary or degenerate polynucleotide sequences could also be used in this embodiment of the present invention.

The present invention further provides fusion proteins encompassing (a) polypeptide molecules comprising  
5 a sequence of amino acid residues as shown in SEQ ID NO. 2 from amino acid residue 1 (Met), 4 (Met), 17 (Gln), 19 (Phe) or 21 (Ala) to amino acid residue 121 (Glu); (b) human paralogs of (a); (c) allelic variants of (a) or (b); (d) polypeptide molecules comprising a sequence of amino  
10 acid residues from amino acid 81 (Asp) or 86 (Phe) to 90 (Met) or 40 (Asp) to 51 (Phe) of SEQ ID NO. 2 and cleavage sites at the 5' and 3' ends thereof; (e) polypeptide molecules comprising a sequence from amino acid 78 (Glu) to amino acid 94 (Ser) or from amino acid 37 (Ser) to  
15 amino acid 54 (His) of SEQ ID NO. 2; or (f) polypeptide molecules comprising a sequence from amino acid 86 (Phe) to amino acid 94 (Ser) of SEQ ID NO. 2 and a cleaving site at the 5' end thereof; and another polypeptide. The other polypeptide may be another neurokinin, a precursor  
20 thereof, a signal peptide to facilitate secretion of the fusion protein or the like.

Generally, precursor proteins, such as zneurok1 polypeptides, are cleaved or processed into active form through the action of prohormone convertases. Zneurok1  
25 polypeptides are believed to be processed by prohormone convertase 1 or prohormone convertase 2, although other prohormone convertase molecules may also serve this purpose. The processed NKB polypeptides are typically amidated at the 5' end through the action of an amidating  
30 enzyme, such as peptidyl-glycyl,  $\alpha$ -amidating monooxygenase (PAM).

The most prevalent cleavage or processing site is a dibasic amino acid prohormone convertase site. Since there are only a few dibasic amino acid combinations,  
35 including KK (lys-lys), RR (arg-arg), RK (arg-lys) and KR (lys-arg), non-dibasic cleavage and processing sites have

also been observed. An example of a non-dibasic site is that found in gastrin (NR). Such cleavage or processing sites may be incorporated into zneurok1 polypeptides, fragments or fusion proteins.

5 A multiple alignment including zneurok1 and murine, rat and bovine NKB precursor proteins is shown in the Figure. All of the aligned NKB precursor proteins include the 10 amino acid NKB protein (amino acid residues 81-90 of SEQ ID NO. 2). Also, the murine and rat NKB  
10 precursor proteins are shorter at the carboxy terminal end than the bovine precursor protein and zneurok1. In addition, the murine and rat NKB precursor proteins do not include the sequence found in both the bovine precursor and in zneurok1 (amino acid residues 30-33 of SEQ ID NO.  
15 2). Also, the Figure shows that zneurok1 lacks a phenylalanine rich region shared by the rat, mouse and bovine NKB precursor proteins (which would be located between residues 45 (Gln) and 46 (Leu) of SEQ ID NO. 2).

The following percent identity figures are  
20 observed for the deduced amino acid sequence of SEQ ID NO. 2 and the other aligned polypeptides shown in the Figure.

	Zneurok1	Rat proNKB	Bovine proNKB	Mouse proNKB
Zneurok1	100	61.7	66	65
Rat proNKB	61.7	100	70	93
Bovine proNKB	66	70	100	75
Mouse proNKB	65	93	75	100

25 The highly conserved amino acids, both within and without the region of high identity, can be used as a tool to identify new family members. For instance, reverse transcription-polymerase chain reaction (RT-PCR) can be used to amplify sequences encoding conserved motifs suggested by the multiple alignment from RNA obtained from

a variety of tissue sources. In particular, the following primers are useful for this purpose:

Amino acid residues 81-86 of SEQ ID NO:2

GAY ATG CAY GAY TTY TT TKNK\_MOUSE (SEQ ID NO:17)  
 5 GAY ATG CAY GAY TTY TT CONSENSUS (SEQ ID NO:18)  
 CTR TAC GTR CTR AAR AA COMPLEMENT (SEQ ID NO:19)

Amino acid residues 87-92 of SEQ ID NO:2

GTN GGN YTN ATG GGN AA TKNK\_MOUSE (SEQ ID NO:20)  
 10 GTN GGN YTN ATG GGN AA CONSENSUS (SEQ ID NO:21)  
 CAN CCN RAN TAC CCN TT COMPLEMENT (SEQ ID NO:22)

Amino acid residues 23-28 of SEQ ID NO:2

TGY GAR GAR CCN CAR GA TKNK\_MOUSE (SEQ ID NO:23)  
 15 TGY RAR GAR HSN CAR GA CONSENSUS (SEQ ID NO:24)  
 ACR YTY CTY DSN GTY CT COMPLEMENT (SEQ ID NO:25)

Amino acid residues 102-107 of SEQ ID NO:2

GAY GRN GRN GAR GAR AA TKNK\_MOUSE (SEQ ID NO:26)  
 20 GAY RTN RWN SAR GAR AA CONSENSUS (SEQ ID NO:27)  
 CTR YAN YWN STY CTY TT COMPLEMENT (SEQ ID NO:28).

Also, the present invention provides probes useful for differentiating species subtypes. Preferred embodiments of this aspect of the present invention  
 25 include the following:

- 1) Amino acids 28-33 of SEQ ID NO. 2 (corresponding to nucleotides 218-235 of SEQ ID NO. 1); and
- 2) Amino acids 44-49 of SEQ ID NO. 2 (corresponding to nucleotides 269-287 of SEQ ID NO. 1).

The present invention also contemplates degenerate probes based upon the polynucleotides described above. Probes corresponding to complements of the  
 35 polynucleotides set forth above are also encompassed.

The present invention also provides polynucleotide molecules, including DNA and RNA molecules, that encode the zneurok1 polypeptides disclosed herein. Those skilled in the art will readily recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. SEQ ID NO:16 is a degenerate DNA sequence that encompasses all DNAs that encode the zneurok1 polypeptide of SEQ ID NO:2. Those skilled in the art will recognize that the degenerate sequence of SEQ ID NO:16 also provides all RNA sequences encoding SEQ ID NO:2 by substituting U (uracil) for T (thymine). Thus, zneurok1 polypeptide-encoding polynucleotides comprising nucleotide 1 to nucleotide 363 of SEQ ID NO:16 and their RNA equivalents are contemplated by the present invention. Additionally, nucleotide 137 to nucleotide 499 of SEQ ID NO:1 corresponds to nucleotide 1 to nucleotide 363 of SEQ ID NO:16; nucleotide 146 to nucleotide 499 of SEQ ID NO:1 corresponds to nucleotide 10 to nucleotide 363 of SEQ ID NO:16; nucleotide 185 to nucleotide 499 of SEQ ID NO:1 corresponds to nucleotide 49 to nucleotide 363 of SEQ ID NO:16; nucleotide 191 to nucleotide 499 of SEQ ID NO:1 corresponds to nucleotide 55 to nucleotide 363 of SEQ ID NO:16; nucleotide 197 to nucleotide 499 of SEQ ID NO:1 corresponds to nucleotide 61 to nucleotide 363 of SEQ ID NO:16; nucleotide 377 to 406 of SEQ ID NO:1 corresponds to nucleotide 241 to nucleotide 270 of SEQ ID NO:16; nucleotide 392 to 406 of SEQ ID NO:1 corresponds to nucleotide 256 to nucleotide 270 of SEQ ID NO:16; nucleotide 368 to 418 of SEQ ID NO:1 corresponds to nucleotide 323 to nucleotide 282 of SEQ ID NO:16; nucleotide 392 to 418 of SEQ ID NO:1 corresponds to nucleotide 256 to nucleotide 282 of SEQ ID NO:16; nucleotide 254 to 289 of SEQ ID NO:1 corresponds to nucleotide 118 to nucleotide 153 of SEQ ID NO:16; and nucleotide 245 to 298 of SEQ ID NO:1 corresponds to



nucleotide 108 to nucleotide 159 of SEQ ID NO:16. Table 1 sets forth the one-letter codes used within SEQ ID NO:16 to denote degenerate nucleotide positions. Table 1 sets forth the one-letter codes used within SEQ ID NO:16 to  
5 denote degenerate nucleotide positions. "Resolutions" are the nucleotides denoted by a code letter. "Complement" indicates the code for the complementary nucleotide(s). For example, the code Y denotes either C (cytosine) or T, and its complement R denotes A (adenine) or G (guanine), A  
10 being complementary to T, and G being complementary to C.

TABLE 1

Nucleotide	Resolution	Nucleotide	Complement
A	A	T	T
C	C	G	G
G	G	C	C
T	T	A	A
R	A G	Y	C T
Y	C T	R	A G
M	A C	K	G T
K	G T	M	A C
S	C G	S	C G
W	A T	W	A T
H	A C T	D	A G T
B	C G T	V	A C G
V	A C G	B	C G T
D	A G T	H	A C T
N	A C G T	N	A C G T

The degenerate codons used in SEQ ID NO:16,  
5 encompassing all possible codons for a given amino acid,  
are set forth in Table 2.

TABLE 2

Amino Acid	One Letter Code	Codons	Degenerate Codon
Cys	C	TGC TGT	TGY
Ser	S	AGC AGT TCA TCC TCG TCT	WSN
Thr	T	ACA ACC ACG ACT	ACN
Pro	P	CCA CCC CCG CCT	CCN
Ala	A	GCA GCC GCG GCT	GCN
Gly	G	GGA GGC GGG GGT	GGN
Asn	N	AAC AAT	AAY
Asp	D	GAC GAT	GAY
Glu	E	GAA GAG	GAR
Gln	Q	CAA CAG	CAR
His	H	CAC CAT	CAY
Arg	R	AGA AGG CGA CGC CGG CGT	MGN
Lys	K	AAA AAG	AAR
Met	M	ATG	ATG
Ile	I	ATA ATC ATT	ATH
Leu	L	CTA CTC CTG CTT TTA TTG	YTN
Val	V	GTA GTC GTG GTT	GTN
Phe	F	TTC TTT	TTY
Tyr	Y	TAC TAT	TAY
Trp	W	TGG	TGG
Ter	.	TAA TAG TGA	TRR
Asn Asp	B		RAY
Glu Gln	Z		SAR
Any	X		NNN

One of ordinary skill in the art will appreciate that some ambiguity is introduced in determining a degenerate codon, representative of all possible codons encoding each amino acid. For example, the degenerate  
5 codon for serine (WSN) can, in some circumstances, encode arginine (AGR), and the degenerate codon for arginine (MGN) can, in some circumstances, encode serine (AGY). A similar relationship exists between codons encoding phenylalanine and leucine. Thus, some polynucleotides  
10 encompassed by the degenerate sequence may encode variant amino acid sequences, but one of ordinary skill in the art can easily identify such variant sequences by reference to the amino acid sequence of SEQ ID NO:2. Variant sequences can be readily tested for functionality as described  
15 herein.

One of ordinary skill in the art will also appreciate that different species can exhibit "preferential codon usage." In general, see, Grantham, et al., Nuc. Acids Res. 8:1893-912, 1980; Haas, et al. Curr.  
20 Biol. 6:315-24, 1996; Wain-Hobson, et al., Gene 13:355-64, 1981; Grosjean and Fiers, Gene 18:199-209, 1982; Holm, Nuc. Acids Res. 14:3075-87, 1986; Ikemura, J. Mol. Biol. 158:573-97, 1982. As used herein, the term "preferential codon usage" or "preferential codons" is a term of art  
25 referring to protein translation codons that are most frequently used in cells of a certain species, thus favoring one or a few representatives of the possible codons encoding each amino acid (See Table 2). For example, the amino acid threonine (Thr) may be encoded by  
30 ACA, ACC, ACG, or ACT, but in mammalian cells ACC is the most commonly used codon; in other species, for example, insect cells, yeast, viruses or bacteria, different Thr codons may be preferential. Preferential codons for a particular species can be introduced into the  
35 polynucleotides of the present invention by a variety of methods known in the art. Introduction of preferential

codon sequences into recombinant DNA can, for example, enhance production of the protein by making protein translation more efficient within a particular cell type or species. Therefore, the degenerate codon sequence disclosed in SEQ ID NO:24 serves as a template for optimizing expression of polynucleotides in various cell types and species commonly used in the art and disclosed herein. Sequences containing preferential codons can be tested and optimized for expression in various species, and tested for functionality as disclosed herein.

Radiation hybrid mapping is a somatic cell genetic technique developed for constructing high-resolution, contiguous maps of mammalian chromosomes (Cox et al., Science 250:245-250, 1990). Partial or full knowledge of a gene's sequence allows the designing of PCR primers suitable for use with chromosomal radiation hybrid mapping panels. Commercially available radiation hybrid mapping panels which cover the entire human genome, such as the Stanford G3 RH Panel and the GeneBridge 4 RH Panel (Research Genetics, Inc., Huntsville, AL), are available. These panels enable rapid, PCR based, chromosomal localizations and ordering of genes, sequence-tagged sites (STSs), and other nonpolymorphic- and polymorphic markers within a region of interest. This includes establishing directly proportional physical distances between newly discovered genes of interest and previously mapped markers. The precise knowledge of a gene's position can be useful in a number of ways including: 1) determining if a sequence is part of an existing contig and obtaining additional surrounding genetic sequences in various forms such as YAC-, BAC- or cDNA clones, 2) providing a possible candidate gene for an inheritable disease which shows linkage to the same chromosomal region, and 3) for cross-referencing model organisms such as mouse which may be beneficial in helping to determine what function a particular gene might have.

The results showed that the gene encoding the zneurok1 polypeptide maps 352.88 cR\_3000 from the top of the human chromosome 12 linkage group on the WICGR radiation hybrid map. Proximal and distal framework  
5 markers were RP\_L41\_1 and CHLC.GATA41A11, respectively. The use of surrounding markers positions the zneurok1 gene in the 12q15 region on the integrated LDB chromosome 12 map.

The present invention also provides reagents  
10 which will find use in diagnostic applications. For example, the zneurok1 gene, a probe comprising zneurok1 DNA or RNA, or a subsequence thereof can be used to determine if the zneurok1 gene is present on chromosome 12 or if a mutation has occurred. Detectable chromosomal  
15 aberrations at the zneurok1 gene locus include, but are not limited to, aneuploidy, gene copy number changes, insertions, deletions, restriction site changes and rearrangements. These aberrations can occur within the coding sequence, within introns, or within flanking  
20 sequences, including upstream promoter and regulatory regions, and may be manifested as physical alterations within a coding sequence or changes in gene expression level.

In general, these diagnostic methods comprise  
25 the steps of (a) obtaining a genetic sample from a patient; (b) incubating the genetic sample with a polynucleotide probe or primer as disclosed above, under conditions wherein the polynucleotide will hybridize to complementary polynucleotide sequence, to produce a first  
30 reaction product; and (iii) comparing the first reaction product to a control reaction product. A difference between the first reaction product and the control reaction product is indicative of a genetic abnormality in the patient. Genetic samples for use within the present  
35 invention include genomic DNA, cDNA, and RNA. The polynucleotide probe or primer can be RNA or DNA, and will

comprise a portion of SEQ ID NO:1, the complement of SEQ ID NO:1, or an RNA equivalent thereof. Suitable assay methods in this regard include molecular genetic techniques known to those in the art, such as restriction fragment length polymorphism (RFLP) analysis, short tandem repeat (STR) analysis employing PCR techniques, ligation chain reaction (Barany, PCR Methods and Applications 1:5-16, 1991), ribonuclease protection assays, and other genetic linkage analysis techniques known in the art (Sambrook et al., ibid.; Ausubel et. al., ibid.; Marian, Chest 108:255-65, 1995). Ribonuclease protection assays (see, e.g., Ausubel et al., ibid., ch. 4) comprise the hybridization of an RNA probe to a patient RNA sample, after which the reaction product (RNA-RNA hybrid) is exposed to RNase. Hybridized regions of the RNA are protected from digestion. Within PCR assays, a patient's genetic sample is incubated with a pair of polynucleotide primers, and the region between the primers is amplified and recovered. Changes in size or amount of recovered product are indicative of mutations in the patient. Another PCR-based technique that can be employed is single strand conformational polymorphism (SSCP) analysis (Hayashi, PCR Methods and Applications 1:34-8, 1991).

An additional aspect of the present invention provides methods for identifying agonists or antagonists of the zneurok1 polypeptides disclosed above, which agonists or antagonists may have valuable properties as discussed further herein. Agonists are generally used in applications where the zneurok1 polypeptide would be useful. In contrast, antagonists are generally used in applications where diminished zneurok1 polypeptide activity is desired.

Within one embodiment, there is provided a method of identifying zneurok1 polypeptide agonists, comprising providing cells responsive thereto, culturing the cells in the presence of a test compound and comparing



the cellular response with the cell cultured in the presence of the zneurok1 polypeptide, and selecting the test compounds for which the cellular response is of the same type.

5           Within another embodiment, there is provided a method of identifying antagonists of zneurok1 polypeptide, comprising providing cells responsive to a zneurok1 polypeptide, culturing a first portion of the cells in the presence of zneurok1 polypeptide, culturing a second  
10 portion of the cells in the presence of the zneurok1 polypeptide and a test compound, and detecting a decrease in a cellular response of the second portion of the cells as compared to the first portion of the cells.

          Within another aspect of the present invention  
15 there is provided a pharmaceutical composition comprising purified zneurok1 in combination with a pharmaceutically acceptable vehicle. Such compositions may be used in modulating inflammation, nociception or emesis and may be formulated for delivery to central, spinal or peripheral  
20 nerves. The zneurok1 polypeptide contains the active polypeptide neurokinin B (NKB, ranging from amino acid 81 to amino acid 90 in SEQ ID NO. 2) flanked by dibasic prohormone convertase cleavage sites. In addition, the zneurok1 polypeptide includes a second putatively active  
25 polypeptide from amino acid 40 (Asp) to 51 (Phe) of SEQ ID NO. 2, flanked by a dibasic cleavage/processing site on the 3' end and a non-dibasic cleavage/processing site at the 5' end thereof.

          A further aspect of the invention provides a  
30 method for studying calcium flux or calcium ion-associated physiological phenomena. Such methods of the present invention comprise incubating calcium-responsive cells in a culture medium comprising zneurok1 polypeptide (or an agonist or antagonist thereof) and observing changes in  
35 calcium flux or a calcium ion-associated physiological phenomena. The impact of zneurok1 polypeptide, fragment,

fusion, agonist or antagonist on intracellular calcium level may be assessed by methods known in the art, such as those described by Dobrzanski et al., Regulatory Peptides 45: 341-52, 1993, and the like.

5           The present invention also provides methods for studying neurokinin receptors. Cells that endogenously express one or more neurokinin receptor types or that are engineered to express such moieties may be used in such methods. Preferably such cells also express or are  
10 engineered to express a reporter construct including a detectable moiety under the control of a regulatory element that is directly or indirectly responsive to NKB binding to a neurokinin receptor. Such methods of the present invention comprise incubating neurokinin receptor-  
15 expressing cells with zneurok1 polypeptide, antibodies directed thereto or agonists or antagonists thereof and observing the cells or expression or activity of the detectable moiety.

          The present invention also provides methods for  
20 studying known prohormone convertases or identifying new prohormone convertases, enzymes which process prohormones and protein precursors. Known prohormone convertases include, but are not limited to, prohormone convertase 3 (PC3), prohormone convertase 2 (PC2), furin, or similar  
25 convertases of the furin family such as prohormone convertase 4 (PC4) and PACE4. Prohormone convertases sometimes exhibit tissue specificity. As a result, zneurok1 polypeptides, which are expressed at high levels in placenta tissue, are likely to be processed by  
30 prohormone convertases exhibiting placenta specificity. In such methods of the present invention, zneurok1 polypeptides or fragments (substrate) may be incubated with known or suspected prohormone convertases (enzyme) to produce neurokinin B (product). The enzyme and substrate  
35 are incubated together or co-expressed in a test cell for a time sufficient to achieve cleavage/processing of the

zneurok1 polypeptide or fragment or fusion thereof. Detection and/or quantification of cleavage products follows, using procedures that are known in the art. For example, the presence of the cleavage products may be established by assays designed to measure neurokinin B function, such as the assays described herein. Alternatively, enzyme kinetics techniques, measuring the rate of cleavage, can be used to study or identify prohormone convertases capable of cleaving zneurok1 polypeptides, fragments or fusion proteins of the present invention.

Polypeptides, fragments, fusion proteins, agonists, antagonists or antibodies of the present invention may also modulate muscle contraction, hormone secretion, DNA synthesis or cell growth, inositol phosphate turnover, arachidonate release, phospholipase-C activation, gastric emptying, human neutrophil activation or ADCC capability, superoxide anion production and the like. Evaluation of these properties can be conducted by known methods, such as those set forth herein.

The impact of zneurok1 polypeptide, fragment, fusion, agonist or antagonist on muscle contraction may be assessed by methods known in the art, such as those described by Smits & Lebeuvre, J. Auton. Pharmacol. 14: 383-92, 1994, Belloli et al., J. Vet. Pharmacol. Therap. 17: 379-83, 1994, Maggi et al., Regulatory Peptides 53: 259-74, 1994, and the like. The impact of zneurok1 polypeptide, fragment, fusion, agonist or antagonist on hormone secretion may be assessed by methods known in the art, such as those for prolactin release described by Henriksen et al., J. of Receptor & Signal Transduction Research 15(1-4): 529-41, 1995, and the like. The impact of zneurok1 polypeptide, fragment, fusion, agonist or antagonist on DNA synthesis or cell growth may be assessed by methods known in the art, such as those described by Dobrzanski et al., Regulatory Peptides 45: 341-52, 1993,

and the like. The impact of zneurok1 polypeptide, fragment, fusion, agonist or antagonist on inositol phosphate turnover may be assessed by methods known in the art, such as those described by Dobrzanski et al.,  
5 Regulatory Peptides 45: 341-52, 1993, and the like.

Also, the impact of zneurok1 polypeptide, fragment, fusion, agonist or antagonist on arachidonate release may be assessed by methods known in the art, such as those described by Dobrzanski et al., Regulatory  
10 Peptides 45: 341-52, 1993, and the like. The impact of zneurok1 polypeptide, fragment, fusion, agonist or antagonist on phospholipase-C activation may be assessed by methods known in the art, such as those described by Dobrzanski et al., Regulatory Peptides 45: 341-52, 1993,  
15 and the like. The impact of zneurok1 polypeptide, fragment, fusion, agonist or antagonist on gastric emptying may be assessed by methods known in the art, such as those described by Varga et al., Eur. J. Pharmacol. 286: 109-112, 1995, and the like. The impact of zneurok1  
20 polypeptide, fragment, fusion, agonist or antagonist on human neutrophil activation and ADCC capability may be assessed by methods known in the art, such as those described by Wozniak et al., Immunology 78: 629-34, 1993, and the like. The impact of zneurok1 polypeptide,  
25 fragment, fusion, agonist or antagonist on superoxide anion production may be assessed by methods known in the art, such as those described by Wozniak et al., Immunology 78: 629-34, 1993, and the like.

Within preferred embodiments of the invention  
30 the isolated polynucleotides will hybridize to similar sized regions of SEQ ID NO. 2, SEQ ID NO. 6, SEQ ID NO. 7, other probe sequences specifically set forth herein or a sequence complementary thereto, under stringent conditions. In general, stringent conditions are selected  
35 to be about 5°C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength and

pH. The  $T_m$  is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typical stringent conditions are those in which the salt concentration is less than about 0.03 M at pH 7 and the temperature is at least about 60°C.

As previously noted, the isolated polynucleotides of the present invention include DNA and RNA. Methods for isolating DNA and RNA are well known in the art. It is generally preferred to isolate RNA from placenta, although DNA can also be prepared using RNA from other tissues or isolated as genomic DNA. Total RNA can be prepared using guanidine HCl extraction followed by isolation by centrifugation in a CsCl gradient (Chirgwin et al., Biochemistry 18:52-94, 1979). Poly (A)<sup>+</sup> RNA is prepared from total RNA using the method of Aviv and Leder (Proc. Natl. Acad. Sci. USA 69:1408-1412, 1972). Complementary DNA (cDNA) is prepared from poly(A)<sup>+</sup> RNA using known methods. Polynucleotides encoding zneurok1 polypeptides are then identified and isolated by, for example, hybridization or PCR.

The present invention further provides counterpart polypeptides and polynucleotides from other species (orthologs). These species include, but are not limited to mammalian, avian, amphibian, reptile, fish, insect and other vertebrate and invertebrate species. Of particular interest are zneurok1 polypeptides from other mammalian species, including porcine, ovine, canine, feline, equine and other primate proteins. Species orthologs of the human proteins can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses the protein. Suitable sources of mRNA can be identified by probing Northern blots with probes designed from the

sequences disclosed herein. A library is then prepared from mRNA of a positive tissue of cell line. A zneurok1 polypeptide-encoding cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequences. A cDNA can also be cloned using the polymerase chain reaction, or PCR (Mullis, U.S. Patent 4,683,202), using primers designed from the sequences disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to zneurok1. Similar techniques can also be applied to the isolation of genomic clones.

A mouse homolog of zneurok1 has also been identified by screening a mouse EST database. The full length nucleotide sequence of the murine zneurok1 homolog was identified (SEQ ID NO:31) by sequencing a clone considered likely to contain it. The deduced amino acid sequence of the murine nucleotide sequence is disclosed in SEQ ID NO:32. Alternate species polypeptides of zneurok1 may have importance therapeutically. It has been demonstrated that in some cases use of a non-native protein, i.e., protein from a different species, can be more potent than the native protein. For example, salmon calcitonin has been shown to be considerably more effective in arresting bone resorption than human forms of calcitonin. There are several hypotheses as to why salmon calcitonin is more potent than human calcitonin in treatment of osteoporosis. These hypotheses include: 1) salmon calcitonin is more resistant to degradation; 2) salmon calcitonin has a lower metabolic clearance rate (MCR); and 3) salmon calcitonin may have a slightly different conformation, resulting in a higher affinity for bone receptor sites. Another example is found in the  $\beta$ -endorphin family (Ho et al., Int. J. Peptide Protein Res.



29:521-4, 1987). Studies have demonstrated that the peripheral opioid activity of camel, horse, turkey and ostrich  $\beta$ -endorphins is greater than that of human  $\beta$ -endorphins when isolated guinea pig ileum was electrostimulated and contractions were measured. Vas deferens from rat, mouse and rabbit were assayed as well. In the rat vas deferens model, camel and horse  $\beta$ -endorphins showed the highest relative potency. Synthesized rat relaxin was as active as human and porcine relaxin in the mouse symphysis pubis assay (Bullesbach and Schwabe, Eur. J. Biochem. 241:533-7, 1996). Thus, the mouse zneurok1 molecule of the present invention may have higher potency than the human endogenous molecule in human cells, tissues and recipients.

Those skilled in the art will recognize that the sequences disclosed in SEQ ID NO:1 and SEQ ID NO:2 represent a single allele of the human zneurok1 gene and polypeptide, and that allelic variation and alternative splicing are expected to occur. Allelic variants can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures. Allelic variants of the DNA sequence shown in SEQ ID NO:1, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins which are allelic variants of SEQ ID NO:2. cDNAs generated from alternatively spliced mRNAs, which retain the properties of the zneurok1 polypeptide are included within the scope of the present invention, as are polypeptides encoded by such cDNAs and mRNAs. Allelic variants and splice variants of these sequences can be cloned by probing cDNA or genomic libraries from different individuals or tissues according to standard procedures known in the art.

The present invention also provides isolated zneurok1 polypeptides that are substantially homologous to



the polypeptides of SEQ ID NO. 2 and their species homologs/orthologs. The term "substantially homologous" is used herein to denote polypeptides having 50%, preferably 60%, more preferably at least 80%, sequence identity to the sequence shown in SEQ ID NO. 2 or its orthologs. Such polypeptides will more preferably be at least 90% identical, and most preferably 95% or more identical to SEQ ID NO. 2 or its orthologs. Percent sequence identity is determined by conventional methods. See, for example, Altschul et al., Bull. Math. Bio. 48: 603-616, 1986 and Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915-10919, 1992. Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "blosum 62" scoring matrix of Henikoff and Henikoff (ibid.) as shown in Table 3 (amino acids are indicated by the standard one-letter codes). The percent identity is then calculated as:

$$\frac{\text{Total number of identical matches}}{[\text{length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences}]} \times 100$$

Table 3

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
5	A	4																		
	R	-1	5																	
	N	-2	0	6																
	D	-2	-2	1	6															
	C	0	-3	-3	-3	9														
	Q	-1	1	0	0	-3	5													
10	E	-1	0	0	2	-4	2	5												
	G	0	-2	0	-1	-3	-2	-2	6											
	H	-2	0	1	-1	-3	0	0	-2	8										
	I	-1	-3	-3	-1	-3	-3	-4	-3	4										
	L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4								
15	K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5							
	M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5						
	F	-2	-3	-3	-3	-2	-3	-3	-1	0	0	-3	0	6						
	P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-1	-2	-4	7					
	S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4			
20	T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-2	-1	1	5			
	W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11	
	Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7

Sequence identity of polynucleotide molecules is determined by similar methods using a ratio as disclosed above.

Substantially homologous proteins and polypeptides are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see Table 4) and other substitutions that do not significantly affect the folding or activity of the protein or polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification (an affinity tag), such as a poly-histidine tract, protein A (Nilsson et al., EMBO J. 4:1075, 1985; Nilsson et al., Methods Enzymol. 198:3, 1991), glutathione S transferase (Smith and Johnson, Gene 67:31, 1988), maltose binding protein (Kellerman and Ferenci, Methods Enzymol. 90:459-463, 1982; Guan et al., Gene 67:21-30, 1987), thioredoxin, ubiquitin, cellulose binding protein, T7 polymerase, or other antigenic epitope or binding domain. See, in general Ford et al., Protein Expression and Purification 2: 95-107, 1991. DNAs encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ; New England Biolabs, Beverly, MA). Polypeptides comprising affinity tags can further comprise a proteolytic cleavage site between the zneurok1 polypeptide and the affinity tag. Preferred such sites include thrombin cleavage sites and factor Xa cleavage sites. The present invention thus includes polypeptides of from 5 to 1000 amino acid residues that comprise a sequence that is at least 60%, preferably at least 80%, and more preferably 90% or more identical to the corresponding region of SEQ ID NO:2.

Table 4Conservative amino acid substitutions

5	Basic:	arginine
		lysine
		histidine
	Acidic:	glutamic acid
		aspartic acid
10	Polar:	glutamine
		asparagine
	Hydrophobic:	leucine
		isoleucine
		valine
15	Aromatic:	phenylalanine
		tryptophan
		tyrosine
	Small:	glycine
		alanine
20		serine
		threonine
		methionine

The proteins of the present invention can also comprise non-naturally occurring amino acid residues. Non-naturally occurring amino acids include, without  
 25 limitation, *trans*-3-methylproline, 2,4-methanoproline, *cis*-4-hydroxyproline, *trans*-4-hydroxyproline, *N*-methylglycine, *allo*-threonine, methylthreonine, hydroxyethylcysteine, hydroxyethylhomocysteine, nitroglutamine, homoglutamine, pipecolic acid, thiazolidine carboxylic acid,  
 30 dehydroproline, 3- and 4-methylproline, 3,3-dimethylproline, *tert*-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, and 4-fluorophenylalanine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues  
 35 into proteins. For example, an *in vitro* system can be employed wherein nonsense mutations are suppressed using

chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is carried out in a cell-free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson et al., J. Am. Chem. Soc. 113:2722, 1991; Ellman et al., Methods Enzymol. 202:301, 1991; Chung et al., Science 259:806-9, 1993; and Chung et al., Proc. Natl. Acad. Sci. USA 90:10145-9, 1993). In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti et al., J. Biol. Chem. 271:19991-8, 1996). Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (e.g., phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (e.g., 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the protein in place of its natural counterpart. See, Koide et al., Biochem. 33:7470-6, 1994. Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro* chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, Protein Sci. 2:395-403, 1993).

A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, non-naturally occurring amino acids, and unnatural amino acids may be substituted for zneurok1 amino acid residues.

Essential amino acids in the zneurok1 polypeptides of the present invention can be identified according to procedures known in the art, such as site-

directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244: 1081-5, 1989). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (e.g., androgen regulation, anti-microbial activity, adhesion modulation or the like) to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton et al., J. Biol. Chem. 271:4699-708, 1996.

10 Sites of ligand-receptor interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et al., Science 255:306-12, 1992; Smith et al., J. Mol. Biol. 224:899-904, 1992; Wlodaver et al., FEBS Lett. 309:59-64, 1992. The identities of essential amino acids can also be inferred from analysis of homologies with

15 related proteins.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (Science 241:53-7, 1988) or Bowie and Sauer (Proc. Natl. Acad. Sci. USA 86:2152-6, 1989). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., Biochem. 30:10832-7, 1991; Ladner et al., U.S. Patent NO: 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis (Derbyshire et al., Gene 46:145, 1986; Ner et al., DNA 7:127, 1988).

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Variants of the disclosed zneurok1 DNA and polypeptide sequences can be generated through DNA shuffling as disclosed by Stemmer, Nature 370:389-91, 1994, Stemmer, Proc. Natl. Acad. Sci. USA 91:10747-51, 5 1994 and WIPO Publication WO 97/20078. Briefly, variant DNAs are generated by *in vitro* homologous recombination by random fragmentation of a parent DNA followed by reassembly using PCR, resulting in randomly introduced point mutations. This technique can be modified by using 10 a family of parent DNAs, such as allelic variants or DNAs from different species, to introduce additional variability into the process. Selection or screening for the desired activity, followed by additional iterations of mutagenesis and assay provides for rapid "evolution" of 15 sequences by selecting for desirable mutations while simultaneously selecting against detrimental changes.

Mutagenesis methods as disclosed above can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides in 20 host cells. Mutagenized DNA molecules that encode active polypeptides (e.g., capable of releasing NKB having NK3 binding activity or calcium ion flux modulation capability) can be recovered from the host cells and rapidly sequenced using modern equipment. These methods 25 allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

Using the methods discussed above, one of 30 ordinary skill in the art can identify and/or prepare a variety of polypeptides that are substantially homologous to residues 17 (Gln), 19 (Phe) or 21 (Ala) to 121 (Glu) of SEQ ID NO. 2 or allelic variants thereof and retain the ability to be cleaved to release NKB having NK3 receptor 35 binding or calcium ion flux modulating properties of the wild-type NKB protein. Such polypeptides may include



additional amino acids, such as affinity tags and the like. Also, such polypeptides may include additional polypeptide segments as generally disclosed above.

The polypeptides of the present invention, including full-length proteins, fragments thereof and fusion proteins, can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multicellular organisms, are preferred. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, and Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987.

In general, a DNA sequence encoding a zneurok1 polypeptide, fragment or fusion of the present invention is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator within an expression vector. The vector will also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

To insure cleavage the neurokinin B polypeptide, cells transfected with expression vectors containing DNA sequences encoding zneurok1 are co-transfected with expression vectors encoding a suitable prohormone convertase, for example furin, PC4, PC2 or PC3. Such sequences are known in the art and can be inserted into expression vectors and transfected into cells as described herein.

To direct a zneurok1 polypeptide, fragment or fusion into the secretory pathway of a host cell, a secretory signal sequence (also known as a signal sequence, leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence may be that of the zneurok1 polypeptide, or may be derived from another secreted protein (e.g., t-PA) or synthesized *de novo*. The secretory signal sequence is joined to the zneurok1-encoding DNA sequence in the correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain signal sequences may be positioned elsewhere in the DNA sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830).

Alternatively, the secretory signal sequence contained in the polypeptides of the present invention is used to direct other polypeptides into the secretory pathway. The present invention provides for such fusion polypeptides. A signal fusion polypeptide can be made wherein a secretory signal sequence derived from residues 1-16, 1-18, 1-20, 4-16, 4-18 or 4-20 of SEQ ID NO:2 is operably linked to another polypeptide using methods known in the art and disclosed herein. The secretory signal sequence contained in the fusion polypeptides of the

present invention is preferably fused amino-terminally to an additional peptide to direct the additional peptide into the secretory pathway. Such constructs have numerous applications known in the art. For example, these novel  
5 secretory signal sequence fusion constructs can direct the secretion of an active component of a normally non-secreted protein, such as a receptor. Such fusions may be used *in vivo* or *in vitro* to direct peptides through the secretory pathway.

10 Cultured mammalian cells are also suitable hosts within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection (Wigler et al., Cell  
14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics  
15 7:603, 1981; Graham and Van der Eb, Virology 52:456, 1973), electroporation (Neumann et al., EMBO J. 1:841-845, 1982), DEAE-dextran mediated transfection (Ausubel et al., eds., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987), liposome-mediated transfection  
20 (Hawley-Nelson et al., Focus 15:73, 1993; Ciccarone et al., Focus 15:80, 1993), and viral vectors (A. Miller and G. Rosman, BioTechniques 7:980-90, 1989; Q. Wang and M. Finer, Nature Med. 2:714-16, 1996). The production of recombinant polypeptides in cultured mammalian cells is  
25 disclosed, for example, by Levinson et al., U.S. Patent No. 4,713,339; Hagen et al., U.S. Patent No. 4,784,950; Palmiter et al., U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134. Preferred cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC  
30 No. CRL 1651), BHK 570 (ATCC No. CRL 10314), 293 (ATCC No. CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) and Chinese hamster ovary (e.g. CHO-K1; ATCC No. CCL 61) cell lines. Additional suitable cell lines are known in the art and available from public depositories such as the  
35 American Type Culture Collection, Rockville, Maryland. In general, strong transcription promoters are suitable, such

as promoters from SV-40 or cytomegalovirus. See, e.g., U.S. Patent No. 4,956,288. Other suitable promoters include those from metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978) and the adenovirus major late promoter.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and are able to pass the gene of interest to their progeny are referred to as "stable transfectants." A preferred selectable marker is a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems may also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (e.g., hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used. Alternative markers that introduce an altered phenotype, such as green fluorescent protein, or cell surface proteins such as CD4, CD8, Class I MHC, placental alkaline phosphatase may be used to sort transfected cells from untransfected cells by such means as FACS sorting or magnetic bead separation technology.

Other higher eukaryotic cells can also be used as hosts, including insect cells, plant cells and avian cells. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by

Sinkar et al., J. Biosci. (Bangalore) 11:47-58, 1987. Transformation of insect cells and production of foreign polypeptides therein is disclosed by Guarino et al., U.S. Patent NO: 5,162,222; Bang et al., U.S. Patent NO: 4,775,624; and WIPO publication WO 94/06463. Insect cells can be infected with recombinant baculovirus, commonly derived from *Autographa californica* nuclear polyhedrosis virus (AcNPV). DNA encoding the zsisg32 polypeptide is inserted into the baculoviral genome in place of the AcNPV polyhedrin gene coding sequence by one of two methods. The first is the traditional method of homologous DNA recombination between wild-type AcNPV and a transfer vector containing the zneurok1 flanked by AcNPV sequences. Suitable insect cells, e.g. SF9 cells, are infected with wild-type AcNPV and transfected with a transfer vector comprising a zneurok1 polynucleotide operably linked to an AcNPV polyhedrin gene promoter, terminator, and flanking sequences. See, King, and Possee, The Baculovirus Expression System: A Laboratory Guide, London, Chapman & Hall; O'Reilly et al., Baculovirus Expression Vectors: A Laboratory Manual, New York, Oxford University Press., 1994; and, Richardson, Ed., Baculovirus Expression Protocols. Methods in Molecular Biology, Totowa, NJ, Humana Press, 1995. Natural recombination within an insect cell will result in a recombinant baculovirus which contains zneurok1 driven by the polyhedrin promoter. Recombinant viral stocks are made by methods commonly used in the art.

The second method of making recombinant baculovirus utilizes a transposon-based system described by Luckow (Luckow, et al., J. Virol. 67:4566-79, 1993). This system is sold in the Bac-to-Bac kit (Life Technologies, Rockville, MD). This system utilizes a transfer vector, pFastBac1™ (Life Technologies) containing a Tn7 transposon to move the DNA encoding the zneurok1 polypeptide into a baculovirus genome maintained in *E.*

*coli* as a large plasmid called a "bacmid." The pFastBac1™ transfer vector utilizes the AcNPV polyhedrin promoter to drive the expression of the gene of interest, in this case *zneurok1*. However, pFastBac1™ can be modified to a considerable degree. The polyhedrin promoter can be removed and substituted with the baculovirus basic protein promoter (also known as Pcor, p6.9 or MP promoter) which is expressed earlier in the baculovirus infection, and has been shown to be advantageous for expressing secreted proteins. See, Hill-Perkins and Possee, J. Gen. Virol. 71:971-6, 1990; Bonning et al., J. Gen. Virol. 75:1551-6, 1994; and, Chazenbalk and Rapoport, J. Biol. Chem. 270:1543-9, 1995. In such transfer vector constructs, a short or long version of the basic protein promoter can be used. Moreover, transfer vectors can be constructed which replace the native *zneurok1* secretory signal sequences with secretory signal sequences derived from insect proteins. For example, a secretory signal sequence from Ecdysteroid Glucosyltransferase (EGT), honey bee Melittin (Invitrogen, Carlsbad, CA), or baculovirus gp67 (PharMingen, San Diego, CA) can be used in constructs to replace the native *zneurok1* secretory signal sequence. In addition, transfer vectors can include an in-frame fusion with DNA encoding an epitope tag at the C- or N-terminus of the expressed *zneurok1* polypeptide, for example, a Glu-Glu epitope tag (Grussenmeyer et al., ibid.). Using techniques known in the art, a transfer vector containing *zneurok1* is transformed into *E. coli*, and screened for bacmids which contain an interrupted *lacZ* gene indicative of recombinant baculovirus. The bacmid DNA containing the recombinant baculovirus genome is isolated, using common techniques, and used to transfect *Spodoptera frugiperda* cells, e.g. Sf9 cells. Recombinant virus that expresses *zneurok1* is subsequently produced. Recombinant viral stocks are made by methods commonly used the art.



The recombinant virus is used to infect host cells, typically a cell line derived from the fall armyworm, *Spodoptera frugiperda*. See, in general, Glick and Pasternak, Molecular Biotechnology: Principles and Applications of Recombinant DNA, ASM Press, Washington, D.C., 1994. Another suitable cell line is the High FiveO™ cell line (Invitrogen) derived from *Trichoplusia ni* (U.S. Patent #5,300,435). Commercially available serum-free media are used to grow and maintain the cells. Suitable media are Sf900 II™ (Life Technologies) or ESF 921™ (Expression Systems) for the Sf9 cells; and Ex-cello405™ (JRH Biosciences, Lenexa, KS) or Express FiveO™ (Life Technologies) for the *T. ni* cells. The cells are grown up from an inoculation density of approximately  $2-5 \times 10^5$  cells to a density of  $1-2 \times 10^6$  cells at which time a recombinant viral stock is added at a multiplicity of infection (MOI) of 0.1 to 10, more typically near 3. The recombinant virus-infected cells typically produce the recombinant zneurok1 polypeptide at 12-72 hours post-infection and secrete it with varying efficiency into the medium. The culture is usually harvested 48 hours post-infection. Centrifugation is used to separate the cells from the medium (supernatant). The supernatant containing the zneurok1 polypeptide is filtered through micropore filters, usually 0.45  $\mu$ m pore size. Procedures used are generally described in available laboratory manuals (King and Possee, *ibid.*; O'Reilly, et al., *ibid.*; Richardson, *ibid.*). Subsequent purification of the zneurok1 polypeptide from the supernatant can be achieved using methods described herein.

Fungal cells, including yeast cells, and particularly cells of the genus *Saccharomyces*, can also be used within the present invention, such as for producing zneurok1 polypeptide fragments or polypeptide fusions. Methods for transforming yeast cells with exogenous DNA



and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent NO: 4,599,311; Kawasaki et al., U.S. Patent NO: 4,931,373; Brake, U.S. Patent NO: 4,870,008; Welch et al., U.S. Patent NO: 5,037,743; and Murray et al., U.S. Patent NO: 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). A preferred vector system for use in yeast is the POT1 vector system disclosed by Kawasaki et al. (U.S. Patent NO: 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S. Patent NO: 4,599,311; Kingsman et al., U.S. Patent NO: 4,615,974; and Bitter, U.S. Patent NO: 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454. Transformation systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, *Pichia methanolica*, *Pichia guilliermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson et al., J. Gen. Microbiol. 132:3459-3465, 1986 and Cregg, U.S. Patent NO: 4,882,279. *Aspergillus* cells may be utilized according to the methods of McKnight et al., U.S. Patent NO: 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino et al., U.S. Patent NO: 5,162,228. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent NO: 4,486,533.

The use of *Pichia methanolica* as host for the production of recombinant proteins is disclosed in WIPO Publications WO 97/17450, WO 97/17451, WO 98/02536, and WO

98/02565. DNA molecules for use in transforming *P. methanolica* will commonly be prepared as double-stranded, circular plasmids, which are preferably linearized prior to transformation. For polypeptide production in *P. methanolica*, it is preferred that the promoter and terminator in the plasmid be that of a *P. methanolica* gene, such as a *P. methanolica* alcohol utilization gene (AUG1 or AUG2). Other useful promoters include those of the dihydroxyacetone synthase (DHAS), formate dehydrogenase (FMD), and catalase (CAT) genes. To facilitate integration of the DNA into the host chromosome, it is preferred to have the entire expression segment of the plasmid flanked at both ends by host DNA sequences. A preferred selectable marker for use in *Pichia methanolica* is a *P. methanolica* ADE2 gene, which encodes phosphoribosyl-5-aminoimidazole carboxylase (AIRC; EC 4.1.1.21), which allows *ade2* host cells to grow in the absence of adenine. For large-scale, industrial processes where it is desirable to minimize the use of methanol, it is preferred to use host cells in which both methanol utilization genes (AUG1 and AUG2) are deleted. For production of secreted proteins, host cells deficient in vacuolar protease genes (PEP4 and PRB1) are preferred. Electroporation is used to facilitate the introduction of a plasmid containing DNA encoding a polypeptide of interest into *P. methanolica* cells. It is preferred to transform *P. methanolica* cells by electroporation using an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm, and a time constant ( $\tau$ ) of from 1 to 40 milliseconds, most preferably about 20 milliseconds.

Prokaryotic host cells, including strains of the bacteria *Escherichia coli*, *Bacillus* and other genera are also useful host cells within the present invention. Techniques for transforming these hosts and expressing foreign DNA sequences cloned therein are well known in the

art (see, e.g., Sambrook et al., ibid.). When expressing a zneurok1 polypeptide in bacteria such as *E. coli*, the polypeptide may be retained in the cytoplasm, typically as insoluble granules, or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or urea. The denatured polypeptide can then be refolded and dimerized by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by dialysis against a buffered saline solution. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic space and recovering the protein, thereby obviating the need for denaturation and refolding.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell. *P. methanolica* cells are cultured in a medium comprising adequate sources of carbon, nitrogen and trace nutrients at a temperature of about 25°C to 35°C. Liquid cultures are provided with sufficient aeration by conventional means, such as shaking

of small flasks or sparging of fermentors. A preferred culture medium for *P. methanolica* is YEPD (2% D-glucose, 2% Bacto™ Peptone (Difco Laboratories, Detroit, MI), 1% Bacto™ yeast extract (Difco Laboratories), 0.004% adenine and 0.006% L-leucine).

Expressed recombinant zneurok1 polypeptides (or chimeric zneurok1 polypeptides) can be purified using fractionation and/or conventional purification methods and media. Ammonium sulfate precipitation and acid or chaotrope extraction may be used for fractionation of samples. Exemplary purification steps may include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable anion exchange media include derivatized dextrans, agarose, cellulose, polyacrylamide, specialty silicas, and the like. PEI, DEAE, QAE and Q derivatives are preferred, with DEAE Fast-Flow Sepharose (Pharmacia, Piscataway, NJ) being particularly preferred. Exemplary chromatographic media include those media derivatized with phenyl, butyl, or octyl groups, such as Phenyl-Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups, hydroxyl groups and/or carbohydrate moieties. Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling chemistries. These and other

solid media are well known and widely used in the art, and are available from commercial suppliers. Methods for binding receptor polypeptides to support media are well known in the art. Selection of a particular method is a matter of routine design and is determined in part by the properties of the chosen support. See, for example, Affinity Chromatography: Principles & Methods, Pharmacia LKB Biotechnology, Uppsala, Sweden, 1988.

The polypeptides of the present invention can be isolated by exploitation of their structural properties. For example, immobilized metal ion adsorption (IMAC) chromatography can be used to purify histidine-rich proteins, or proteins containing a His-tag. Briefly, a gel is first charged with divalent metal ions to form a chelate (E. Sulkowski, Trends in Biochem. 3:1-7, 1985). Histidine-rich proteins will be adsorbed to this matrix with differing affinities, depending upon the metal ion used, and will be eluted by competitive elution, lowering the pH, or use of strong chelating agents. Other methods of purification include purification of glycosylated proteins by lectin affinity chromatography and ion exchange chromatography (Methods in Enzymol., Vol. 182, "Guide to Protein Purification", M. Deutscher, (ed.), Acad. Press, San Diego, 1990, pp.529-39). Within other embodiments of the invention, a fusion of the polypeptide of interest and an affinity tag (e.g., polyhistidine, Glu-Glu, FLAG, maltose-binding protein, an immunoglobulin domain) may be constructed to facilitate purification.

Protein refolding (and optionally reoxidation) procedures may be advantageously used. It is preferred to purify the protein to >80% purity, more preferably to >90% purity, even more preferably >95% purity, and particularly preferred is a pharmaceutically pure state, that is greater than 99.9% pure with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents.

Preferably, a purified protein is substantially free of other proteins, particularly other proteins of animal origin.

5 Zneurok1 polypeptides or fragments thereof may also be prepared through chemical synthesis. Zneurok1 polypeptides may be monomers or multimers; glycosylated or non-glycosylated; pegylated or non-pegylated; and may or may not include an initial methionine amino acid residue.

10 A zneurok1 ligand-binding polypeptide, such as NK3 receptors, can also be used for purification of ligand. The polypeptide is immobilized on a solid support, such as beads of agarose, cross-linked agarose, glass, cellulosic resins, silica-based resins, polystyrene, cross-linked polyacrylamide, or like  
15 materials that are stable under the conditions of use. Methods for linking polypeptides to solid supports are known in the art, and include amine chemistry, cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, and hydrazide  
20 activation. The resulting medium will generally be configured in the form of a column, and fluids containing ligand are passed through the column one or more times to allow ligand to bind to the receptor polypeptide. The ligand is then eluted using changes in salt concentration,  
25 chaotropic agents (guanidine HCl), or pH to disrupt ligand-receptor binding.

An assay system that uses a ligand-binding receptor (or an antibody, one member of a complement/anti-complement pair) or a binding fragment thereof, and a  
30 commercially available biosensor instrument (BIAcore™, Pharmacia Biosensor, Piscataway, NJ) may be advantageously employed. Such receptor, antibody, member of a complement/anti-complement pair or fragment is immobilized onto the surface of a receptor chip. Use of this  
35 instrument is disclosed by Karlsson, J. Immunol. Methods 145:229-40, 1991 and Cunningham and Wells, J. Mol. Biol.



234:554-63, 1993. A receptor, antibody, member or fragment is covalently attached, using amine or sulfhydryl chemistry, to dextran fibers that are attached to gold film within the flow cell. A test sample is passed  
5 through the cell. If a ligand, epitope, or opposite member of the complement/anti-complement pair is present in the sample, it will bind to the immobilized receptor, antibody or member, respectively, causing a change in the refractive index of the medium, which is detected as a  
10 change in surface plasmon resonance of the gold film. This system allows the determination of on- and off-rates, from which binding affinity can be calculated, and assessment of stoichiometry of binding.

Ligand-binding receptor polypeptides can also be  
15 used within other assay systems known in the art. Such systems include Scatchard analysis for determination of binding affinity (see Scatchard, Ann. NY Acad. Sci. 51: 660-72, 1949) and calorimetric assays (Cunningham et al., Science 253:545-48, 1991; Cunningham et al., Science  
20 245:821-25, 1991).

Zneurok1 can be measured *in vitro* using cultured cells or *in vivo* by administering molecules of the claimed invention to the appropriate animal model. For instance, zneurok1 transfected or co-transfected expression host  
25 cells may be embedded in an alginate environment and injected (implanted) into recipient animals. Alginate-poly-L-lysine microencapsulation, permselective membrane encapsulation and diffusion chambers have been described as a means to entrap transfected mammalian cells or  
30 primary mammalian cells. These types of non-immunogenic "encapsulations" or microenvironments permit the transfer of nutrients into the microenvironment, and also permit the diffusion of proteins and other macromolecules secreted or released by the captured cells across the  
35 environmental barrier to the recipient animal. Most importantly, the capsules or microenvironments mask and



shield the foreign, embedded cells from the recipient animal's immune response. Such microenvironments can extend the life of the injected cells from a few hours or days (naked cells) to several weeks (embedded cells).

5           Alginate threads provide a simple and quick means for generating embedded cells. The materials needed to generate the alginate threads are readily available and relatively inexpensive. Once made, the alginate threads are relatively strong and durable, both *in vitro* and,  
10 based on data obtained using the threads, *in vivo*. The alginate threads are easily manipulable and the methodology is scalable for preparation of numerous threads. In an exemplary procedure, 3% alginate is prepared in sterile H<sub>2</sub>O, and sterile filtered. Just prior  
15 to preparation of alginate threads, the alginate solution is again filtered. An approximately 50% cell suspension (containing about  $5 \times 10^5$  to about  $5 \times 10^7$  cells/ml) is mixed with the 3% alginate solution. One ml of the alginate/cell suspension is extruded into a 100 mM sterile  
20 filtered CaCl<sub>2</sub> solution over a time period of ~15 min, forming a "thread". The extruded thread is then transferred into a solution of 50 mM CaCl<sub>2</sub>, and then into a solution of 25 mM CaCl<sub>2</sub>. The thread is then rinsed with deionized water before coating the thread by incubating in  
25 a 0.01% solution of poly-L-lysine. Finally, the thread is rinsed with Lactated Ringer's Solution and drawn from solution into a syringe barrel (without needle attached). A large bore needle is then attached to the syringe, and the thread is intraperitoneally injected into a recipient  
30 in a minimal volume of the Lactated Ringer's Solution.

          An alternative *in vivo* approach for assaying proteins of the present invention involves viral delivery systems. Exemplary viruses for this purpose include adenovirus, herpesvirus, vaccinia virus and adeno-  
35 associated virus (AAV). Adenovirus, a double-stranded DNA virus, is currently the best studied gene transfer vector

for delivery of heterologous nucleic acid (for a review, see Becker et al., Meth. Cell Biol. 43:161-89, 1994; and Douglas and Curiel, Science & Medicine 4:44-53, 1997). The adenovirus system offers several advantages:  
5 adenovirus can (i) accommodate relatively large DNA inserts; (ii) be grown to high-titer; (iii) infect a broad range of mammalian cell types; and (iv) be used with a large number of available vectors containing different promoters. Also, because adenoviruses are stable in the  
10 bloodstream, they can be administered by intravenous injection.

By deleting portions of the adenovirus genome, larger inserts (up to 7 kb) of heterologous DNA can be accommodated. These inserts can be incorporated into the  
15 viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. In an exemplary system, the essential E1 gene has been deleted from the viral vector, and the virus will not replicate unless the E1 gene is provided by the host cell (the human  
20 293 cell line is exemplary). When intravenously administered to intact animals, adenovirus primarily targets the liver. If the adenoviral delivery system has an E1 gene deletion, the virus cannot replicate in the host cells. However, the host's tissue (e.g., liver) will  
25 express and process (and, if a secretory signal sequence is present, secrete) the heterologous protein. Secreted proteins will enter the circulation in the highly vascularized liver, and effects on the infected animal can be determined.

30 The adenovirus system can also be used for protein production *in vitro*. By culturing adenovirus-infected non-293 cells under conditions where the cells are not rapidly dividing, the cells can produce proteins for extended periods of time. For instance, BHK cells are  
35 grown to confluence in cell factories, then exposed to the adenoviral vector encoding the secreted protein of

interest. The cells are then grown under serum-free conditions, which allows infected cells to survive for several weeks without significant cell division. Alternatively, adenovirus vector infected 293S cells can  
5 be grown in suspension culture at relatively high cell density to produce significant amounts of protein (see Garnier et al., Cytotechnol. 15:145-55, 1994). With either protocol, an expressed, secreted heterologous protein can be repeatedly isolated from the cell culture  
10 supernatant. Within the infected 293S cell production protocol, non-secreted proteins may also be effectively obtained.

Zneurok1 polypeptides can also be used to prepare antibodies that specifically bind to zneurok1  
15 epitopes, peptides or polypeptides. Antibodies generated from this immune response can be isolated and purified as described herein. Methods for preparing and isolating polyclonal and monoclonal antibodies are well known in the art. See, for example, Current Protocols in Immunology,  
20 Cooligan, et al. (eds.), National Institutes of Health, John Wiley and Sons, Inc., 1995; Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989; and Hurrell, J. G. R., Ed., Monoclonal Hybridoma Antibodies: Techniques and  
25 Applications, CRC Press, Inc., Boca Raton, FL, 1982.

As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from inoculating a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice,  
30 hamsters, guinea pigs and rats as well as transgenic animals such as transgenic sheep, cows, goats or pigs. Antibodies may also be expressed in yeast and fungi in modified forms as well as in mammalian and insect cells. The zneurok1 polypeptide or a fragment thereof serves as  
35 an antigen (immunogen) to inoculate an animal or elicit an immune response. Suitable antigens would include the

zneurok1 polypeptide encoded by SEQ ID NO:2 from amino acid residue 17, 19 or 21 to 121 of SEQ ID NO:2, or a contiguous 9-121 amino acid residue fragment thereof. The immunogenicity of a zneurok1 polypeptide may be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of zneurok1 or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like", such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

As used herein, the term "antibodies" includes polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, and antigen-binding fragments, such as F(ab')<sub>2</sub> and Fab proteolytic fragments. Genetically engineered intact antibodies or fragments, such as chimeric antibodies, Fv fragments, single chain antibodies and the like, as well as synthetic antigen-binding peptides and polypeptides, are also included. Non-human antibodies may be humanized by grafting non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by replacement of exposed residues, wherein the result is a "veneered" antibody). In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper binding characteristics. Through humanizing antibodies, biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans is reduced. Human antibodies can also be made in mice

having a humanized humoral immune system (Mendez et al., Nat. Genet. 14:146-56, 1997).

Alternative techniques for generating or selecting antibodies useful herein include *in vitro* exposure of lymphocytes to zneurokl protein or peptide, and selection of antibody display libraries, in phage or similar vectors (for instance, through use of immobilized or labeled zneurokl protein or peptide). Mutagenesis methods discussed herein, in particular domain shuffling, can be used to generate and mature antibodies.

The antibodies of the current invention, or fragments thereof, can be used to direct molecules to a specific target. For example, as T-bodies, chimeric receptors combining antibody recognition with T cell effector function, (Eshhar et al., Springer Semin. Immunopathol. 18:199-209, 1996; Eshhar, Cancer Immunol. Immunother. 45:131-6, 1997). Intrabodies, engineered single-chain antibodies expressed inside the cell and having high affinity and specificity for intracellular targets. Such molecules have use in gene therapy and treatment of infectious diseases (Marasco, Immunotechnology 1:1-19, 1995; Marasco et al., Gene Ther. 4:11-5, 1997; Rondon and Marasco, Annu. Rev. Microbiol. 51:257-83, 1997 and Mhashilkar et al., J. Virol. 71:6486-94, 1997). Diabodies, bispecific non-covalent dimers of scFv antibodies useful for immunodiagnosis and therapeutically. In addition they can be constructed in bacteria (Holliger et al., Proc. Natl. Acad. Sci. USA 90:6444-48, 1993).

Antibodies herein specifically bind if they bind to a zneurokl polypeptide, peptide or epitope with a binding affinity ( $K_a$ ) of  $10^6 \text{ M}^{-1}$  or greater, preferably  $10^7 \text{ M}^{-1}$  or greater, more preferably  $10^8 \text{ M}^{-1}$  or greater, and most preferably  $10^9 \text{ M}^{-1}$  or greater. The binding affinity of an antibody can be readily determined by one

of ordinary skill in the art, for example, by Scatchard analysis (Scatchard, ibid.).

Genes encoding polypeptides having potential zneurok1 polypeptide binding domains, "binding proteins",  
5 can be obtained by screening random or directed peptide libraries displayed on phage (phage display) or on bacteria, such as *E. coli*. Nucleotide sequences encoding the polypeptides can be obtained in a number of ways, such as through random mutagenesis and random polynucleotide  
10 synthesis. Alternatively, constrained phage display libraries can also be produced. These peptide display libraries can be used to screen for peptides which interact with a known target which can be a protein or polypeptide, such as a ligand or receptor, a biological or  
15 synthetic macromolecule, or organic or inorganic substances. Techniques for creating and screening such peptide display libraries are known in the art (Ladner et al., US Patent NO. 5,223,409; Ladner et al., US Patent NO. 4,946,778; Ladner et al., US Patent NO. 5,403,484 and  
20 Ladner et al., US Patent NO. 5,571,698) and peptide display libraries and kits for screening such libraries are available commercially, for instance from Clontech (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA) and Pharmacia LKB  
25 Biotechnology Inc. (Piscataway, NJ). Peptide display libraries can be screened using the zneurok1 sequences disclosed herein to identify proteins which bind to zneurok1. These "binding proteins" which interact with zneurok1 polypeptides can be used essentially like an  
30 antibody, for tagging cells; for isolating homolog polypeptides by affinity purification; directly or indirectly conjugated to drugs, toxins, radionuclides and the like. These binding proteins can also be used in analytical methods such as for screening expression  
35 libraries and neutralizing activity. The binding proteins can also be used for diagnostic assays for determining



circulating levels of polypeptides; for detecting or quantitating soluble polypeptides as marker of underlying pathology or disease. To increase the half-life of these binding proteins, they can be conjugated. Their  
5 biological properties may be modified by dimerizing or multimerizing for use as agonists or antagonists.

A variety of assays known to those skilled in the art can be utilized to detect antibodies and/or binding proteins which specifically bind to zneurok1  
10 proteins or peptides. Exemplary assays are described in detail in Antibodies: A Laboratory Manual, Harlow and Lane (Eds.), Cold Spring Harbor Laboratory Press, 1988. Representative examples of such assays include: concurrent immunoelectrophoresis, radioimmunoassay, radioimmuno-  
15 precipitation, enzyme-linked immunosorbent assay (ELISA), dot blot or Western blot assay, inhibition or competition assay, and sandwich assay. In addition, antibodies can be screened for binding to wild-type versus mutant zneurok1 protein or polypeptide.

20 Antibodies to zneurok1 polypeptides may be used for tagging cells that express zneurok1 polypeptide; for isolating zneurok1 polypeptides by affinity purification; for diagnostic assays for determining circulating levels of zneurok1 polypeptides; for detecting or quantitating  
25 soluble zneurok1 as marker of underlying pathology or disease; in analytical methods employing FACS; for screening expression libraries; for generating anti-idiotypic antibodies; and as neutralizing antibodies or as antagonists to block zneurok1 activity *in vitro* and *in*  
30 *vivo*. Suitable direct tags or labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like; indirect tags or labels may feature use of biotin-avidin or other complement/anti-  
35 complement pairs as intermediates. Antibodies herein may also be directly or indirectly conjugated to drugs,



toxins, radionuclides and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications.

Molecules of the present invention can be used to identify and isolate receptors involved in NKB  
5 function. For example, polypeptide fragments of the present invention that present NKB in or near its physiological conformation can be immobilized on a column and membrane preparations run over the column (Immobilized Affinity Ligand Techniques, Hermanson et al., eds.,  
10 Academic Press, San Diego, CA, 1992, pp.195-202). Proteins and peptides can also be radiolabeled (Methods in Enzymol., vol. 182, "Guide to Protein Purification", M. Deutscher, ed., Acad. Press, San Diego, 1990, 721-737) or photoaffinity labeled (Brunner et al., Ann. Rev. Biochem.  
15 62:483-514, 1993 and Fedan et al., Biochem. Pharmacol. 33:1167-1180, 1984) and specific cell-surface proteins can be identified.

For pharmaceutical use, the proteins of the present invention are formulated for parenteral,  
20 particularly intravenous or subcutaneous, delivery according to conventional methods. Intravenous administration will be by bolus injection or infusion over a typical period of one to several hours. In general, pharmaceutical formulations will include a zneurokl  
25 protein in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water or the like. Formulations may further include one or more excipients, preservatives, solubilizers, buffering agents, albumin to prevent protein loss on vial surfaces,  
30 etc. Methods of formulation are well known in the art and are disclosed, for example, in Remington: The Science and Practice of Pharmacy, Gennaro, ed., Mack Publishing Co., Easton, PA, 19th ed., 1995. Therapeutic doses will generally be determined by the clinician according to  
35 accepted standards, taking into account the nature and severity of the condition to be treated, patient traits,

etc. Determination of dose is within the level of ordinary skill in the art.

Polynucleotides encoding zneurok1 polypeptides are useful within gene therapy applications where it is  
5 desired to increase or inhibit zneurok1 activity. If a mammal has a mutated or absent zneurok1 gene, the zneurok1 gene can be introduced into the cells of the mammal. In one embodiment, a gene encoding a zneurok1 polypeptide is introduced *in vivo* in a viral vector. Such vectors  
10 include an attenuated or defective DNA virus, such as, but not limited to, herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral  
15 genes, are preferred. A defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Examples of particular vectors include, but  
20 are not limited to, a defective herpes simplex virus 1 (HSV1) vector (Kaplitt et al., Molec. Cell. Neurosci. 2:320-30, 1991); an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al., J. Clin. Invest. 90:626-30, 1992; and a defective adeno-  
25 associated virus vector (Samulski et al., J. Virol. 61:3096-101, 1987; Samulski et al., J. Virol. 63:3822-8, 1989).

In another embodiment, a zneurok1 gene can be introduced in a retroviral vector, e.g., as described in  
30 Anderson et al., U.S. Patent NO: 5,399,346; Mann et al. Cell 33:153, 1983; Temin et al., U.S. Patent NO: 4,650,764; Temin et al., U.S. Patent NO: 4,980,289; Markowitz et al., J. Virol. 62:1120, 1988; Temin et al., U.S. Patent NO: 5,124,263; International Patent  
35 Publication NO: WO 95/07358, published March 16, 1995 by Dougherty et al.; and Kuo et al., Blood 82:845, 1993.

Alternatively, the vector can be introduced by lipofection in vivo using liposomes. Synthetic cationic lipids can be used to prepare liposomes for in vivo transfection of a gene encoding a marker (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413-7, 1987; Mackey et al., Proc. Natl. Acad. Sci. USA 85:8027-31, 1988). The use of lipofection to introduce exogenous genes into specific organs in vivo has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. More particularly, directing transfection to particular cells represents one area of benefit. For instance, directing transfection to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting. Targeted peptides (e.g., hormones or neurotransmitters), proteins, such as antibodies, or non-peptide molecules can be coupled to liposomes chemically.

It is possible to remove the target cells from the body; to introduce the vector as a naked DNA plasmid; and then to re-implant the transformed cells into the body. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, e.g., transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun or use of a DNA vector transporter. See, e.g., Wu et al., J. Biol. Chem. 267:963-7, 1992; Wu et al., J. Biol. Chem. 263:14621-4, 1988.

Antisense methodology can be used to inhibit zneurok1 gene transcription, such as to inhibit cell proliferation in vivo. Polynucleotides that are complementary to a segment of a zneurok1-encoding polynucleotide (e.g., a polynucleotide as set forth in SEQ ID NO:1) are designed to bind to zneurok1-encoding mRNA

and to inhibit translation of such mRNA. Such antisense polynucleotides are used to inhibit expression of zneurok1 polypeptide-encoding genes in cell culture or in a subject.

5 Transgenic mice, engineered to express the zneurok1 gene, and mice that exhibit a complete absence of zneurok1 gene function, referred to as "knockout mice" (Snouwaert et al., Science 257:1083, 1992), may also be generated (Lowell et al., Nature 366:740-42, 1993). These  
10 mice may be employed to study the zneurok1 gene and the protein encoded thereby in an *in vivo* system.

The invention is further illustrated by the following non-limiting examples.

15

#### Example 1

##### Extension of EST Sequence

The novel zneurok1 polypeptide-encoding  
20 polynucleotides of the present invention were initially identified by selecting an EST from an EST database, predicting a protein sequence based thereupon, and searching known sequence databases for the secreted protein that is most homologous to predicted protein based  
25 on the EST. ESTs that potentially encode proteins having biologically interesting homology to known secreted proteins were identified for further study. A single EST sequence was discovered and predicted to be incorporate neurokinin B, a known modulator of calcium ion  
30 concentration. See, for example, Kotani et al., Proc. Natl. Acad. Sci (USA) 83: 7074-8, 1986. To identify the corresponding cDNA, a clone considered likely to contain the entire coding sequence was used for sequencing. Using an Invitrogen S.N.A.P.<sup>TM</sup> Miniprep kit (Invitrogen, Corp.,  
35 San Diego, CA) according to manufacturer's instructions a 5 ml overnight culture in LB + 50 µg/ml ampicillin was

prepared. The template was sequenced on an ABIPRISM™ model 377 DNA sequencer (Perkin-Elmer Cetus Corp., Norwalk, Ct.) using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Cetus Corp.) according to manufacturer's instructions. Oligonucleotides ZC447 (SEQ ID NO.4), ZC976 (SEQ ID NO.5) for lacZ-containing vectors were used as sequencing primers. Oligonucleotides ZC12973 (SEQ ID NO.6), ZC12974 (SEQ ID NO.7) were used to complete the sequence from the clone. Sequencing reactions were carried out in a Hybaid OmniGene Temperature Cycling System (National Labnet Co., Woodbridge, NY). SEQUENCHER™ 3.0 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI) was used for data analysis. The resulting 812 bp sequence is disclosed in SEQ ID NO. 1. Comparison of the originally derived EST sequence with the sequence represented in SEQ ID NO. 1 showed that there were 11 base pair differences and 16 base pair insertions which dramatically altered the amino acid sequence from that predicted based upon the EST sequence. Note that two of the base pair differences were from unknown "N" residues in the EST sequence to known residues in SEQ ID NO. 1.

A mouse homolog of zneurok1 was also identified by searching known mouse sequence databases for sequences homologous to the zneurok1 sequences disclosed herein. One such EST was discovered and a clone considered likely to contain the complete sequence used for sequencing as described above. The complete the nucleotide sequence is disclosed in SEQ ID NO:29 and the deduced amino acid sequence in SEQ ID NO:30.

#### Example 2 Tissue Distribution

35

Northerns were performed using Human Multiple Tissue Blots from Clontech (Palo Alto, CA). An

approximately 1 kb DNA fragment was excised from the vector containing the identified EST by restriction digestion using Eco RI and Xho I. The fragment was purified using a QIAquick gel extraction kit (Qiagen Inc., Chatsworth, California). The DNA probe was radioactively labeled with <sup>32</sup>P using a random priming MEGAPRIME® DNA labeling system (Amersham, Arlington Heights, IL) according to manufacturer's specifications. The probe was purified using a NUCTRAP push column (Stratagene Cloning Systems, La Jolla, CA). EXPRESSHYB (Clontech, Palo Alto, CA) solution was used for prehybridization and as a hybridizing solution for the Northern blots. Hybridization took place at 65°C overnight using 1x10<sup>6</sup> cpm/ml of labeled probe. The blots were then washed in 2X SSC and 0.05% SDS at 25°C for 45 minutes, followed by a one hour wash in 1X SSC and 0.1% SDS at 50°C. The blots were exposed to film for 4 hours, and the resulting blots exhibited high background. Consequently, the blots were washed for an additional 1½ hours in 1X SSC and 0.1% SDS at 55°C. A 1.2 kb transcript was expressed very highly in the placenta and was detected at much lower levels in brain, small intestine, stomach and spinal cord.

### Example 3

#### Chromosomal Mapping

Zneurok1 polypeptide was mapped to chromosome 12 using the commercially available "GeneBridge 4 Radiation Hybrid Panel" (Research Genetics, Inc., Huntsville, AL). The GeneBridge 4 Radiation Hybrid Panel contains PCRable DNAs from each of 93 radiation hybrid clones, plus two control DNAs (the HFL donor and the A23 recipient). A publicly available WWW server (<http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl>) allows mapping relative to the Whitehead Institute/MIT Center for Genome Research's radiation hybrid map of the human genome (the "WICGR"



radiation hybrid map) which was constructed with the GeneBridge 4 Radiation Hybrid Panel.

For the mapping of the zneurok1 gene with the "GeneBridge 4 Radiation Hybrid Panel", 20  $\mu$ l reactions were set up in a 96-well microtiter plate (Stratagene, La Jolla, CA) and used in a "RoboCycler Gradient 96" thermal cycler (Stratagene). Each of the 95 PCR reactions consisted of 2  $\mu$ l 10X KlenTaq PCR reaction buffer (Clontech), 1.6  $\mu$ l dNTPs mix (2.5 mM each, PERKIN-ELMER, Foster City, CA), 1  $\mu$ l sense primer, ZC 13,894 (SEQ ID NO. 8), 1  $\mu$ l antisense primer, ZC 13,895 (SEQ ID NO. 9), 2  $\mu$ l RediLoad (Research Genetics, Inc.), 0.4  $\mu$ l 50X Advantage KlenTaq Polymerase Mix (Clontech Laboratories, Inc.), 25 ng of DNA from an individual hybrid clone or control and ddH<sub>2</sub>O for a total volume of 20  $\mu$ l. The reactions were overlaid with an equal amount of mineral oil and sealed. The PCR cycler conditions were as follows: an initial 1 cycle 5 minute denaturation at 95°C, 35 cycles of a 1 minute denaturation at 95°C, 1 minute annealing at 60°C and 1.5 minute extension at 72°C, followed by a final 1 cycle extension of 7 minutes at 72°C. The reactions were separated by electrophoresis on a 3% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME).

The results showed that the gene encoding the zneurok1 polypeptide maps 352.88 cR\_3000 from the top of the human chromosome 12 linkage group on the WICGR radiation hybrid map. Proximal and distal framework markers were RP\_L41\_1 and CHLC.GATA41A11, respectively. The use of surrounding markers positions the zneurok1 gene in the 12q15 region on the integrated LDB chromosome 12 map (The Genetic Location Database, University of Southampton, WWW server: [http://cedar.genetics.soton.ac.uk/public\\_html/](http://cedar.genetics.soton.ac.uk/public_html/)).



Construction of ZNEUROK-1 Mammalian Expression Vectors  
ZNEUROK-1CF/pZP9, ZNEUROK-1NF#1/pZP9 and ZNEUROK-  
1NF#2/pZP9

5           Three expression vectors were prepared for the  
ZNEUROK-1 polypeptide, ZNEUROK-1CF/pZP9, designed to  
express a C-terminal FLAG-tagged zneruok-1 polypeptide and  
ZNEUROK-1NF#1/pZP9 and ZNEUROK-1NF#2/pZP9 wherein the  
constructs are designed to express a ZNEUROK-1 polypeptide  
10 with a N-terminal FLAG tag (SEQ ID NO:10). Construct #1  
encodes a polypeptide wherein the amino acid sequence of  
the mature polypeptide begins at amino acid residue 21  
(Ala) of SEQ ID NO:2. Construct #2 encodes a polypeptide  
wherein the mature polypeptide begins at amino acid  
15 residue 19 (Phe) of SEQ ID NO:2.

ZNEUROK-1CF/pZP9

A 363 bp PCR generated ZNEUROK-1 DNA fragment  
was created using ZC13634 (SEQ ID NO:11) and ZC13635 (SEQ  
20 ID NO:12) as PCR primers and the template described in  
Example 1 above. This will encode a full length zneurok1  
polypeptide including the signal sequence from amino acid  
residue 1 to 20 of SEQ ID NO:2. The PCR reaction was  
incubated at 94°C for 30 seconds, and then run for 10  
25 cycles of 30 seconds at 94°C and 30 seconds at 50°C,  
followed by 25 cycles at 94°C for 30 seconds and 65°C for  
90 seconds followed by a 10 minute extension at 72°C. The  
resultant PCR product was then run on a 0.1% TAE agarose  
gel. A band of the predicted size, 264 bp, was excised  
30 and the DNA was purified from the gel with a QUIAQUICK®  
column (Qiagen) according the manufacturer's instructions.  
The DNA was digested with the restriction enzymes Xho I  
and Bam HI, followed by extraction and precipitated.

The excised DNA was subcloned into plasmid  
35 CF/pZP9 which had been cut with Xho I and Bam HI. The  
ZNEUROK-1/CFpZP9 expression vector uses the native  
ZNEUROK-1 signal peptide, and the FLAG epitope (SEQ ID

NO:10) is attached at the C-terminus as a purification aid. Plasmid CF/pZP9 (deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD) is a mammalian expression vector containing an expression  
5 cassette having the mouse metallothionein-1 promoter, multiple restriction sites for insertion of coding sequences, a sequence encoding the flag peptide (SEQ ID NO:7), a stop codon and a human growth hormone terminator. The plasmid also has an *E. coli* origin of replication, a  
10 mammalian selectable marker expression unit having an SV40 promoter, enhancer and origin of replication, a DHFR gene and the SV40 terminator.

#### ZNEUROK-1NF#1/pZP9

15 A 320 bp PCR generated ZNEUROK-1 DNA fragment was created in accordance with the procedure set forth above using ZC13685 (SEQ ID NO:13) and ZC13693 (SEQ ID NO:14) as PCR primers. This will encode a mature polypeptide beginning at amino acid residue 21 (Ala) of  
20 SEQ ID NO:2. The purified PCR fragment was digested with the restriction enzymes Bam HI and Xho I, followed by extraction and precipitation.

The excised ZNEUROK-1 DNA was subcloned into plasmid NF/pZP9 which had been cut with Bam HI and Xho I.  
25 The ZNEUROK-1/NFpZP9 expression vector incorporates the TPA leader and attaches the FLAG epitope (SEQ ID NO:10) to the N-terminal of the ZNEUROK-1 polypeptide-encoding polynucleotide sequence. Plasmid NF/pZP9 (deposited at the American Type Culture Collection, 12301 Parklawn  
30 Drive, Rockville, MD) is a mammalian expression vector containing an expression cassette having the mouse metallothionein-1 promoter, a TPA leader peptide followed by the sequence encoding the FLAG peptide (SEQ ID NO:10), multiple restriction sites for insertion of coding  
35 sequences, and a human growth hormone terminator. The plasmid also contains an *E. coli* origin of replication, a

mammalian selectable marker expression unit having an SV40 promoter, enhancer and origin of replication, a DHFR gene and the SV40 terminator.

5                   ZNEUROK-1NF#2/pZP9

                  A 326 bp PCR generated ZNEUROK-1 DNA fragment was created in accordance with the procedure set forth above using ZC13685 (SEQ ID NO:13) and ZC13694 (SEQ ID NO:15) as PCR primers. This will encode a mature  
10 polypeptide beginning at amino acid residue 19 (Phe) of SEQ ID NO:2. The purified PCR fragment was digested with the restriction enzymes Bam HI and Xho I, followed by extraction and precipitation and was ligated in the expression vector zZP9 as described above.

15                   Ten nanograms of the restriction digested Zneruok-1 inserts and 20 ng of the corresponding vectors were ligated at room temperature for 4 hours. One microliter of each ligation reaction was independently electroporated into DH10B competent cells (GIBCO BRL,  
20 Gaithersburg, MD) according to manufacturer's direction and plated onto LB plates containing 50 mg/ml ampicillin, and incubated overnight. Colonies were screened by PCR as described above. The primers for ZNEUROK-1CF/pZP9 were, ZC13634 (SEQ ID NO:11) and ZC13635 (SEQ ID NO:12), for  
25 ZNEUROK-1NF#1/pZP9 the primers were ZC13685 (SEQ ID NO:12) and ZC13693 (SEQ ID NO:14) and for ZNEUROK-1NF#2/pZP9 the primers were ZC13685 (SEQ ID NO:12) and ZC13694 (SEQ ID NO:11). The PCR reaction was incubated at 94°C for 90 seconds, and then run for 35 cycles of 94°C for 30 seconds  
30 and 69°C for 90 seconds, followed by 25 cycles at 94°C for 30 seconds and 65°C for 90 seconds followed by a 10 minute extension at 72°C. The insert sequence of positive clones, 264bp fragment for ZNEUROK-1CF, a 320 bp fragment for ZNEUROK-1#1/NF and a 326 bp fragment for ZNEUROK-  
35 1#1/NF were verified by sequence analysis. A large scale

plasmid preparation was done using a QIAGEN<sup>®</sup> Maxi prep kit (Qiagen) according to manufacturer's instructions.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been  
5 described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

- (i) APPLICANT: ZymoGenetics, Inc.  
1201 Eastlake Avenue E.  
Seattle  
WA  
USA  
98102
- (ii) TITLE OF THE INVENTION: NEUROKININ B PRECURSORS
- (iii) NUMBER OF SEQUENCES: 30
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: ZymoGenetics
  - (B) STREET: 1201 Eastlake Ave. E.
  - (C) CITY: Seattle
  - (D) STATE: WA
  - (E) COUNTRY: USA
  - (F) ZIP: 98102
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Diskette
  - (B) COMPUTER: IBM Compatible
  - (C) OPERATING SYSTEM: DOS
  - (D) SOFTWARE: FastSEQ for Windows Version 2.0
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Lingenfelter, Susan E
  - (B) REGISTRATION NUMBER: 41,156
  - (C) REFERENCE/DOCKET NUMBER: 97-26PC
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 206-442-6675

(B) TELEFAX: 206-442-6678

(C) TELEX:

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 812 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 137...499

(D) OTHER INFORMATION:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATTCGGCAC GAGGTTTCTC TCTTTGCAGG AGCACCGGCA GCACCAGTGT GTGAGGGGAG	60
CAGGCAGCGG TCCTAGCCAG TTCCTTGATC CTGCCAGACC ACCCAGCCCC CGGCACAGAG	120
CTGCTCCACA GGCACC ATG AGG ATC ATG CTG CTA TTC ACA GCC ATC CTG GCC	172
Met Arg Ile Met Leu Leu Phe Thr Ala Ile Leu Ala	
1 5 10	
 TTC AGC CTA GCT CAG AGC TTT GGG GCT GTC TGT AAG GAG CCA CAG GAG	220
Phe Ser Leu Ala Gln Ser Phe Gly Ala Val Cys Lys Glu Pro Gln Glu	
15 20 25	
 GAG GTG GTT CCT GGC GGG GGC CGC AGC AAG AGG GAT CCA GAT CTC TAC	268
Glu Val Val Pro Gly Gly Gly Arg Ser Lys Arg Asp Pro Asp Leu Tyr	
30 35 40	
 CAG CTG CTC CAG AGA CTC TTC AAA AGC CAC TCA TCT CTG GAG GGA TTG	316
Gln Leu Leu Gln Arg Leu Phe Lys Ser His Ser Ser Leu Glu Gly Leu	
45 50 55 60	
 CTC AAA GCC CTG AGC CAG GCT AGC ACA GAT CCT AAG GAA TCA ACA TCT	364
Leu Lys Ala Leu Ser Gln Ala Ser Thr Asp Pro Lys Glu Ser Thr Ser	
65 70 75	

76

CCC GAG AAA CGT GAC ATG CAT GAC TTC TTT GTG GGA CTT ATG GGC AAG 412  
 Pro Glu Lys Arg Asp Met His Asp Phe Phe Val Gly Leu Met Gly Lys  
                   80                                  85                                  90

AGG AGC GTC CAG CCA GAC TCT CCT ACG GAT GTG AAT CAA GAG AAC GTC 460  
 Arg Ser Val Gln Pro Asp Ser Pro Thr Asp Val Asn Gln Glu Asn Val  
                   95                                  100                                  105

CCC AGC TTT GGC ATC CTC AAG TAT CCC CCG AGA GCA GAA TAGGTACTCC AC 511  
 Pro Ser Phe Gly Ile Leu Lys Tyr Pro Pro Arg Ala Glu  
                   110                                  115                                  120

TTCCGGACTC CTGGACTGCA TTAGGAAGAC CTCTTTCCCT GTCCCAATCC CCAGGTGCGC 571  
 ACGCTCCTGT TACCCTTTCT CTTCCCTGTT CTTGTAACAT TCTTGTGCTT TGACTCCTTC 631  
 TCCATCTTTT CTACCTGACC CTGGTGTGGA AACTGCATAG TGAATATCCC CAACCCCAAT 691  
 GGGCATTGAC TGTAGAATAC CCTAGAGTTC CTGTAGTGTC CTACATTAAA AATATAATGT 751  
 CTCTCTCTAT TCCTCAACAA TAAAGGATTT TTGCATATGA AAAAAAAAAA AAAAAAAAAA 811  
 C 812

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 121 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Arg Ile Met Leu Leu Phe Thr Ala Ile Leu Ala Phe Ser Leu Ala  
   1                  5                                  10                                  15  
 Gln Ser Phe Gly Ala Val Cys Lys Glu Pro Gln Glu Glu Val Val Pro  
                   20                                  25                                  30  
 Gly Gly Gly Arg Ser Lys Arg Asp Pro Asp Leu Tyr Gln Leu Leu Gln  
                   35                                  40                                  45  
 Arg Leu Phe Lys Ser His Ser Ser Leu Glu Gly Leu Leu Lys Ala Leu  
                   50                                  55                                  60  
 Ser Gln Ala Ser Thr Asp Pro Lys Glu Ser Thr Ser Pro Glu Lys Arg  
 65                                  70                                  75                                  80  
 Asp Met His Asp Phe Phe Val Gly Leu Met Gly Lys Arg Ser Val Gln  
                   85                                  90                                  95



77

Pro Asp Ser Pro Thr Asp Val Asn Gln Glu Asn Val Pro Ser Phe Gly  
100 105 110  
Ile Leu Lys Tyr Pro Pro Arg Ala Glu  
115 120

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Phe Xaa Gly Leu Met  
1 5

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: ZC447

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TAACAATTTC ACACAGG

17

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: ZC976

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGTTGTAAAA CGACGGCC

18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC12973

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGAGTACCTA TTCTGCTCTC G

21

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC12974

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAGGGATCCA GATCTCTACC

20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC13894

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TCTTTGTGGG ACTTATGG

18

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC13895

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTACCTATTC TGCTCTCG

18

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Asp Tyr Lys Asp Asp Asp Asp Lys  
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC13634

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTACTCGAGA TGAGGATCAT GCTGCTA

27

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC13635

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTAGGATCCT TCTGCTCTCG GGGGATA

27

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC13685

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTACTCGAGG TGGAGTACCT ATTCTGC

27

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: ZC13693

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTAGGATCCG CTGTCTGTAA GGAGCCA

27

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC13694

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TTAGGATCCT TTGGGGCTGT CTGTAAG

27

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 363 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ATGMGNATHA	TGYTNYTNTT	YACNGCNATH	YTNGCNTTYW	SNYTNGCNCA	RWSNTTYGGN	60
GCNGTNTGYA	ARGARCCNCA	RGARGARGTN	GTNCCNGGNG	GNGGNMGNWS	NAARMGNGAY	120
CCNGAYYTNT	AYCARYTNYT	NCARMGNYTN	TTYAARWSNC	AYWSNWSNYT	NGARGGNYTN	180
YTNAARGCNY	TNWSNCARGC	NWSNACNGAY	CCNAARGARW	SNACNWSNCC	NGARAARMGN	240
GAYATGCAYG	AYTTYTTYGT	NGGNYTNATG	GGNAARMGNW	SNGTNCARCC	NGAYWSNCCN	300
ACNGAYGTNA	AYCARGARAA	YGTNCCNWSN	TTYGGNATHY	TNAARTAYCC	NCCNMGNGCN	360
GAR						363

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GAYATGCAYG AYT TYTT

17

## (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GAYATGCAYG AYT TYTT

17

## (2) INFORMATION FOR SEQ ID NO:19:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CTR TACGTRC TRA ARAA

17

## (2) INFORMATION FOR SEQ ID NO:20:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GTNGGNYTNA TGGGNAA

17

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTNGGNYTNA TGGGNAA

17

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CANCCNRANT ACCCNTT

17

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single



(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TGYGARGARC CNCARGA

17

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TGYRARGARH SNCARGA

17

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ACRYTYCTYD SNGTYCT

17

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GAYGRNGRNG ARGARAA

17

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GAYRTNRWNS ARGARAA

17

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CTRYANYWNS TYCTYTT

17

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 698 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 74...348

(D) OTHER INFORMATION.

(xi) SEQUENCE DESCRIPTION. SEQ ID NO:29.

GAATTCGGCA TGAGGCAGCC AGCTCCCTGA TCCTGTCAGC TATCCAGCTC CACAGCTTTG	60
TCCTTCAGGC ACC ATG AGG AGC GCC ATG CTG TTT GCG GCT GTC CTC GCC	109
Met Arg Ser Ala Met Leu Phe Ala Ala Val Leu Ala	
1 5 10	
CTC AGC TTG GCT TGG ACC TTC GGG GCT GTG TGT GAG GAG CCA CAG GGG	157
Leu Ser Leu Ala Trp Thr Phe Gly Ala Val Cys Glu Glu Pro Gln Gly	
15 20 25	
CAG GGA GGG AGG CTC AGT AAG GAC TCT GAT CTC TAT CAG CTG CCT CCG	205
Gln Gly Gly Arg Leu Ser Lys Asp Ser Asp Leu Tyr Gln Leu Pro Pro	
30 35 40	
TCC CTG CTT CGG AGA CTC TAC GAC AGC CGC CCT GTC TCT CTG GAA GGA	253
Ser Leu Leu Arg Arg Leu Tyr Asp Ser Arg Pro Val Ser Leu Glu Gly	
45 50 55 60	
TTG CTG AAA GTG CTG AGC AAG GCT AGC GTG GGA CCA AAG GAG ACA TCA	301
Leu Leu Lys Val Leu Ser Lys Ala Ser Val Gly Pro Lys Glu Thr Ser	
65 70 75	
CTT CCA CAG AAA CGT GAC ATG CAC GAC TTC TTT GTG GGA CTT ATG GG CA	350
Leu Pro Gln Lys Arg Asp Met His Asp Phe Phe Val Gly Leu Met Gly	
80 85 90	
AGAGGAACAG CCAACCAGAC ACTCCCACCG ACGTGGTTGA AGAGAACACC CCCAGCTTTG	410
GCATCCTCAA ATAATCCCC AGTACAGAAA AGCACTCTAC CCCTGGACCC CGGATTGCAT	470
CATAAAGCAG TGCCTGCTCC TGCTTCCTCT TCTCCTCCCC TGCTGTAAAG GTCCTCCTGT	530
TGGCTCCCTT CCTACTCTGC ATAGAAGCTG CATATGAACA GCCTCAACCC CATAGCAATT	590
ATGGTTTCTG TAGTGTCTCTG CATTAAAAAT GCTATCTCTC TTCGTCAACA ATAAAGGGTT	650
TTTACAAAGG AAAAAAAAAA AAAAAAAAAA AAAAATTTC GCGGCCGC	698

(2) INFORMATION FOR SEQ ID NO:30.

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 92 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION. SEQ ID NO:30

Met	Arg	Ser	Ala	Met	Leu	Phe	Ala	Ala	Val	Leu	Ala	Leu	Ser	Leu	Ala
1				5					10					15	
Trp	Thr	Phe	Gly	Ala	Val	Cys	Glu	Glu	Pro	Gln	Gly	Gln	Gly	Gly	Arg
			20					25					30		
Leu	Ser	Lys	Asp	Ser	Asp	Leu	Tyr	Gln	Leu	Pro	Pro	Ser	Leu	Leu	Arg
		35					40					45			
Arg	Leu	Tyr	Asp	Ser	Arg	Pro	Val	Ser	Leu	Glu	Gly	Leu	Leu	Lys	Val
	50					55				60					
Leu	Ser	Lys	Ala	Ser	Val	Gly	Pro	Lys	Glu	Thr	Ser	Leu	Pro	Gln	Lys
65					70					75				80	
Arg	Asp	Met	His	Asp	Phe	Phe	Val	Gly	Leu	Met	Gly				
				85					90						

## WHAT IS CLAIMED IS:

1. An isolated polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of amino acid residue 17, 19 or 21 to amino acid residue 121 of SEQ ID NO. 2, and wherein said polypeptide releases a neurokinin B polypeptide in the presence of a prohormone convertase capable of cleaving dibasic amino acids.
2. An isolated polypeptide, according to claim 1, having the amino acid sequence of amino acid residue 17, 19 or 21 to amino acid residue 121 of SEQ ID NO. 2.
3. An isolated polypeptide, according to claim 1, having the amino acid sequence of amino acid residue 1 or 4 to amino acid residue 121 of SEQ ID NO:2.
4. A pharmaceutical composition comprising a polypeptide according to claim 1, in combination with a pharmaceutically acceptable vehicle.
5. An antibody that specifically binds to an epitope of a polypeptide of SEQ ID NO:2.
6. An anti-idiotypic antibody of an antibody which specifically binds to an epitope of a polypeptide of SEQ ID NO:2.
7. A binding protein which specifically binds to an epitope of a polypeptide of SEQ ID NO:2.
8. A fusion protein consisting essentially of a first portion and a second portion joined by a peptide bond, said first portion consisting essentially of a polypeptide according to claim 1, and said second portion consisting essentially of a second polypeptide.

9. A fusion protein comprising a secretory signal sequence selected from the group consisting of:

- (a) amino acid residues 1-16 of SEQ ID NO:2;
- (b) amino acid residues 1-18 of SEQ ID NO:2;
- (c) amino acid residues 1-20 of SEQ ID NO:2;
- (d) amino acid residues 4-16 of SEQ ID NO:2;
- (e) amino acid residues 4-18 of SEQ ID NO:2; and
- (f) amino acid residues 4-10 of SEQ ID NO:2;

wherein said secretory signal sequence is operably linked to an additional polypeptide.

10. An isolated polynucleotide molecule encoding a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of amino acid residue 17, 19 or 21 to amino acid residue 121 of SEQ ID NO. 2, and wherein said polypeptide releases a neurokinin B polypeptide in the presence of a prohormone convertase capable of cleaving dibasic amino acids.

11. An isolated polynucleotide molecule according to claim 10, encoding a polypeptide having the amino acid sequence of amino acid residue 17, 19 or 21 to amino acid residue 121 of SEQ ID NO. 2.

12. An isolated polynucleotide molecule according to claim 10, encoding a polypeptide having the amino acid sequence of amino acid residue 1 or 4 to amino acid residue 121 of SEQ ID NO:2.

13. An isolated polynucleotide molecule according to claim 10, wherein said polynucleotide molecule is selected from the group consisting of:

- (a) a polynucleotide molecule having the sequence of nucleotide 137 to nucleotide 812 of SEQ ID NO:1;
- (b) a polynucleotide molecule having the sequence of nucleotide 146 to nucleotide 812 of SEQ ID NO:1;

(c) a polynucleotide molecule having the sequence of nucleotide 188 to nucleotide 812 of SEQ ID NO:1;

(d) a polynucleotide molecule having the sequence of nucleotide 191 to nucleotide 812 of SEQ ID NO:1;

(e) a polynucleotide molecule having the sequence of nucleotide 197 to nucleotide 812 of SEQ ID NO:1;

(f) degenerate nucleotide sequences of (a), (b), (c), (d) or (e); and

molecules complementary to (a), (b), (c), (d), (e) or (f).

14. A polynucleotide encoding a human zneurok1 polypeptide fragment.

15. A polynucleotide according to claim 14, wherein said polynucleotide further encodes human zneurok1 polypeptide fragments having native or engineered N-terminal and a C-terminal proteolytic cleavage sites cleavable by a prohormone convertase.

16. A polynucleotide according to claim 14 wherein said polynucleotide is selected from the group consisting of:

- (a) nucleotide 377 to 406 of SEQ ID NO:1;
- (b) nucleotide 392 to 406 of SEQ ID NO:1;
- (c) nucleotide 368 to 418 of SEQ ID NO:1;
- (d) nucleotide 392 to 418 of SEQ ID NO:1;
- (e) nucleotide 254 to 289 of SEQ ID NO:1; and
- (e) nucleotide 245 to 298 of SEQ ID NO:1;.

17. A polynucleotide according to claim 15, wherein said N-terminal and C-terminal cleavage sites are independently dibasic cleavage sites selected from the group consisting of:

- (a) lys-lys;
- (b) arg-arg;
- (c) lys-arg; and



(d) arg-lys.

18. An isolated polynucleotide molecule encoding a fusion protein consisting essentially of a first portion and a second portion joined by a peptide bond, said first portion comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of residue 17, 19 or 21 to residue 121 of SEQ ID NO. 2, and wherein said polypeptide releases a neurokinin B polypeptide in the presence of a prohormone convertase capable of cleaving dibasic amino acids; and said second portion consisting essentially of a second polypeptide.

19. An isolated polynucleotide molecule encoding a fusion protein comprising a secretory signal sequence selected from the group consisting of:

- (a) amino acid residues 1-16 of SEQ ID NO:2;
- (b) amino acid residues 1-18 of SEQ ID NO:2;
- (c) amino acid residues 1-20 of SEQ ID NO:2;
- (d) amino acid residues 4-16 of SEQ ID NO:2;
- (e) amino acid residues 4-18 of SEQ ID NO:2; and
- (f) amino acid residues 4-10 of SEQ ID NO:2;

wherein said secretory signal sequence is operably linked to an additional polypeptide.

20. An expression vector comprising the following operably linked elements:

- a transcription promoter;
- a DNA segment encoding a polypeptide according to claim 1; and
- a transcription terminator.

21. An expression vector according to claim 20 wherein said DNA segment further encodes a secretory signal sequence operably linked to said polypeptide.

22. An expression vector according the claim 21, wherein said secretory signal sequence is selected from the group consisting of:

- (a) amino acid residues 1-16 of SEQ ID NO:2;
- (b) amino acid residues 1-18 of SEQ ID NO:2;
- (c) amino acid residues 1-20 of SEQ ID NO:2;
- (d) amino acid residues 4-16 of SEQ ID NO:2;
- (e) amino acid residues 4-18 of SEQ ID NO:2; and
- (f) amino acid residues 4-10 of SEQ ID NO:2.

23. A cultured cell into which has been introduced an expression vector according to claim 20;

wherein said cell expresses said polypeptide encoded by said DNA segment.

24. A method of producing a polypeptide comprising: culturing a cell into which has been introduced an expression vector according to claim 20;

whereby said cell expresses said polypeptide encoded by said DNA segment; and recovering said expressed polypeptide.

25. A method for detecting a genetic abnormality in a patient, comprising:

obtaining a genetic sample from a patient;

incubating the genetic sample with a polynucleotide comprising at least 14 contiguous nucleotides of SEQ ID NO:1 or the complement of SEQ ID NO:1, under conditions wherein said polynucleotide will hybridize to complementary polynucleotide sequence, to produce a first reaction product;

comparing said first reaction product to a control reaction product, wherein a difference between said first reaction product and said control reaction product is indicative of a genetic abnormality in the patient.

TKNK_MOUSE	MRSAMLFAAV	LALSLAWTFG	AVCEEPQEQ.	...GGRLSKD	SDLYQLPPSL	; 46
TKNK_RAT	MRSAMLFAAV	LALSLAWTFG	AACEEPQEQ.	...GGRLSKD	SDLSLLPPPL	; 46
TKNK_BOVIN	MRSTLLFAVI	LALSSARSLG	AVCEESQEQV	VPGGGHSKKD	SNLYQLPPSL	; 50
zneurok1	MRIMLLFTAI	LAFSLAQSG	AVCKEPQEEV	VPGGGRSKRD	PDLYQ....L	; 46
TKNK_MOUSE	LRRLYDSRPV	SLEGLLKVLS	KASVGPKETS	LPQKRDMHDF	FVGLMGKRNS	; 96
TKNK_RAT	LRRLYDSRSI	SLEGLLKVLS	KASVGPKETS	LPQKRDMHDF	FVGLMGKRNS	; 96
TKNK_BOVIN	LRRLYDSRVV	SLDGLLKMLS	KASVGPKESP	LPQKRDMHDF	FVGLMGKRNL	; 100
zneurok1	LQRLFKSHS.	SLEGLLKALS	QASTDPKEST	SPEKRDMHDF	FVGLMGKRSV	; 95
TKNK_MOUSE	QPDTPDVVE	ENTPSFGILK	~~~~~			; 116
TKNK_RAT	QPDTPADVVE	ENTPSFGVLK	~~~~~			; 116
TKNK_BOVIN	QPDTPVDINQ	ENIPSGFTFK	YPPSVE			; 126
zneurok1	QPDSPTDVNQ	ENVPSFGILK	YPPRAE			; 121

FIG. 1

# INTERNATIONAL SEARCH REPORT

Inter. Appl. Application No  
PCT/US 98/10842

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N15/62 C07K7/22 C07K16/18 C07K19/00  
C12N5/10 A61K38/04 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	EMBL databank Accession number AA004764 Hillier L et al. 25-JUL-1996 (Rel. 48, Created) "similar to neurokinin B" XP002076990 see the whole document	1-25
Y	LANG S ET AL: "Neurochemical characterization of preprotachykinin B(50-79) immunoreactivity in the rat" REGULATORY PEPTIDES, 1995, 57, 183-192, XP002076987 see abstract see page 191; figure 1	1-25

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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

9 September 1998

Date of mailing of the international search report

22/09/1998

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# INTERNATIONAL SEARCH REPORT

Inter. Appl. Application No  
PCT/US 98/10842

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	<p>BONNER TI ET AL: "A cDNA encoding the precursor of the rat neuropeptide, neurokinin B."  BRAIN RES, SEP 1987, 388 (3) P243-9, XP002076988  NETHERLANDS  see the whole document  ---</p>	1-25
A	<p>US 5 410 019 A (COY DAVID H ET AL)  25 April 1995  see abstract; table 1  ---</p>	1-25
A	<p>KOTANI H ET AL: "Structure and gene organization of bovine neuromedin K precursor."  PROC NATL ACAD SCI U S A, SEP 1986, 83 (18) P7074-8, XP002076989  UNITED STATES  cited in the application  see the whole document  -----</p>	1-25

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Inter. Application No

PCT/US 98/10842

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